

**IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE**

GENENTECH, INC.,

Plaintiff,

v.

AMGEN INC.,

Defendant.

C.A. No. 18-924-CFC

JURY TRIAL DEMANDED

THIRD AMENDED COMPLAINT

Plaintiff Genentech, Inc. (“Genentech”) brings this Third Amended Complaint against Defendant Amgen Inc. (“Amgen”) to address Amgen’s infringement of patents relating to Genentech’s groundbreaking breast cancer drug Herceptin®.

NATURE OF THE CASE

1. Breast cancer is a serious disease affecting over 2.8 million women in the United States. Approximately 20-25% of those women suffer from “HER2-positive” breast cancer. This is a particularly aggressive form of the disease characterized by overexpression of human epidermal growth factor receptor 2 (i.e., “HER2”) proteins due to excessive HER2 gene amplification.

2. In the early 1990s, a diagnosis of HER2-positive breast cancer was effectively a death sentence: patients had an average life expectancy of only 18 months. The quality of life for those patients was markedly poor—the disease rapidly metastasized (*i.e.*, spread to other parts of the body). The only available treatments were invasive and disfiguring surgery and chemotherapeutic drugs with harsh side effects, and those treatments added little to the patient’s life span.

3. The treatment of HER2-positive breast cancer, and the lives of millions of women suffering from the disease, changed dramatically with Genentech's development of Herceptin[®]. Herceptin[®] was the first drug of its kind—an antibody called trastuzumab that specifically targeted the biological mechanism that makes HER2-positive breast cancer such an aggressive form of the disease.

4. Although the scientific community was initially skeptical that such an antibody-based therapy could work, Genentech's specific methods of using Herceptin[®] proved remarkably effective. Indeed, after Genentech revealed the results of its clinical studies, the scientific community hailed Herceptin[®] as “the beginning of a whole new wave of biological drugs that modulate the causes of cancer”¹ and a sign that “the whole field of cancer research has turned a corner.”²

5. Since FDA approval of Herceptin[®] in 1998, Genentech has worked diligently to develop new methods of using Herceptin[®]—including improved dosing schedules and broader indications—to expand access to therapy and improve the quality of life for millions of patients worldwide. This research has greatly expanded the number of patients who are able to benefit from Herceptin[®]. To further expand access to this lifesaving drug, Genentech also provides Herceptin[®] free of charge to patients who are uninsured or cannot afford treatment and assists with out-of-pocket prescription-related expenses. All told, Genentech has spent over two decades, and billions of dollars, developing Herceptin[®] into the life-saving drug it is today.

6. Genentech's groundbreaking work developing Herceptin[®] was the result of years of research from a group of talented scientists. The United States Patent and Trademark Office

¹ Gina Kolata and Lawrence M. Fisher, *Drugs to Fight Breast Cancer Near Approval*, NEW YORK TIMES (FRONT PAGE) (Sept. 3, 1998).

² Robert Langreth, *Breast-Cancer Drug Is Backed by FDA Panel*, Wall Street J. (Sept. 3, 1998).

recognized that innovative work by granting Genentech numerous patents claiming Herceptin[®], its manufacture, and its use.

7. Seeking to profit from the success of Genentech's innovations, Amgen sought FDA approval of a biosimilar version of Herceptin[®] called ABP 980 (trastuzumab-anns). The FDA approved ABP 980, which Amgen markets under the tradename Kanjinti, on June 13, 2019 for the same label indications and usage as Herceptin[®]. In fact, Amgen relied upon Genentech's own studies demonstrating the safety and efficacy of Herceptin[®] to obtain approval of its biosimilar product.

8. In 2010, Congress provided a pathway for resolving patent disputes relating to biosimilar products through the Biologics Price Competition and Innovation Act ("BPCIA"). Amgen initially purported to follow the process outlined in the BPCIA, which requires biosimilar applicants and innovator companies to exchange certain information concerning the biosimilar product and the patents that may be infringed by the manufacture and sale of the biosimilar product. *See* 42 U.S.C. § 262(l).

9. Genentech thus brings this action for infringement pursuant to 35 U.S.C. § 271(e)(2) based upon Amgen's submission of its Abbreviated Biologics License Application ("aBLA") for ABP 980. Genentech also seeks a judgment of infringement under 35 U.S.C. § 271(a), (b), and (g) that Amgen's manufacture, use, offer to sell, sale, or importation into the United States of Amgen's biosimilar product has infringed the patents described below. Genentech also seeks a declaratory judgment pursuant to 42 U.S.C. § 262(l)(9) and 28 U.S.C. § 2201 that the manufacture, use, offer to sell, sale, or importation into the United States of Amgen's biosimilar product would infringe the patents described below. Pursuant to 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283,

Genentech also seeks a preliminary and/or permanent injunction barring Amgen's manufacture, use, offer to sell, sale, or importation of its biosimilar product prior to the expiration of those patents. Genentech also seeks monetary damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of Genentech's patents, and any further relief as this Court may deem just and proper.

PARTIES

10. Plaintiff Genentech is a corporation organized and existing under the laws of the State of Delaware with its corporate headquarters at 1 DNA Way, South San Francisco, California 94080.

11. Genentech was founded in 1976 and for four decades has been at the forefront of innovation in the field of therapeutic biotechnology. Today, Genentech employs a large number of researchers, scientists, and post-doctoral staff members who routinely publish in top peer-reviewed journals and are among the leaders in total citations to their work by researchers. Genentech currently markets numerous approved pharmaceutical and biologic drugs for a range of serious or life-threatening medical conditions, including various forms of cancer, heart attacks, strokes, rheumatoid arthritis, and respiratory diseases.

12. Upon information and belief, Defendant Amgen is a company organized and existing under the laws of the State of Delaware with its principal place of business located at One Amgen Center Drive, Thousand Oaks, California 91320.

13. Amgen is, among other things, engaged in the development of biologic drugs, including a biosimilar version of Genentech's Herceptin[®] product, ABP 980 ("Amgen's aBLA product"). Upon information and belief, Amgen's aBLA product is or will be distributed and sold in the State of Delaware and throughout the United States.

JURISDICTION AND VENUE

14. This action arises under the BPCIA, 42 U.S.C. § 262(*l*) and the Patent Laws of the United States, Title 35, United States Code, and the Declaratory Judgment Act, 28 U.S.C. §§ 2201-2202. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331, 1332, and 1338.

15. Venue is proper with respect to Amgen in this Court pursuant to 28 U.S.C. §§ 1391 and 1400(b) because Amgen is incorporated in Delaware.

16. This Court has personal jurisdiction over Amgen because it is incorporated in Delaware. In addition, among other things, Amgen has been approved by the FDA to market its aBLA product and has announced that its aBLA product is now available to customers in the United States, including in Delaware.

THE PARTIES' EXCHANGES UNDER THE BPCIA

17. On July 31, 2017, Amgen announced that it had submitted an aBLA for ABP 980 to the FDA seeking approval for the commercial manufacture, use, offer for sale, or sale of the Amgen aBLA product, a biosimilar version of trastuzumab, which is subject to BLA No. 103792 to Genentech.³

18. The FDA accepted Amgen's aBLA for review on September 26, 2017.

19. On October 16, 2017, Amgen provided Genentech with a copy of Amgen's aBLA, which included a small amount of manufacturing information.

20. On November 3, 2017, Amgen provided Genentech with additional manufacturing information regarding Amgen's aBLA product.

³ <http://www.amgen.com/media/news-releases/2017/07/amgen-and-allergan-submit-biosimilar-biologics-license-application-for-abp-980-to-us-food-and-drug-administration/>

21. Genentech responded on November 20, 2017, to identify deficiencies in Amgen's production of manufacturing information and request specific information concerning the manufacture of Amgen's biosimilar product. Amgen provided additional manufacturing information on December 1, 2017, and December 4, 2017, but did not satisfy its disclosure obligations. Genentech then responded on December 15, 2017, to explain that Amgen's production was deficient in that it failed to provide all of the requested information in contravention of 42 U.S.C. § 262(l)(2).

22. Amgen did not disclose all of the information relevant to establishing whether the manufacture of Amgen's aBLA product will infringe each of the patents identified on Genentech's operative list pursuant to 42 U.S.C. § 262(l)(3)(A), despite Genentech's request that Amgen provide sufficient "other information that describes the process or processes used to manufacture" as required by 42 U.S.C. § 262(l)(A). Amgen's failure to provide sufficient information under those circumstances justifies Genentech's contention that manufacturing Amgen's aBLA product will infringe such patents.

23. Despite Amgen's non-compliance (and without waiving Genentech's objection to such non-compliance), Genentech provided its operative list of 36 patents pursuant to 42 U.S.C. § 262(l)(3)(A) on December 15, 2017.

24. Amgen replied on December 20, 2017, to assert its position that it had complied with its disclosure obligations based on Amgen's earlier production of its aBLA and two manufacturing documents.

25. Genentech responded on December 27, 2017, to reiterate that Amgen's production was insufficient to provide Genentech with a complete understanding of Amgen's trastuzumab manufacturing process.

26. Amgen replied on February 1, 2018, with an additional supplemental production.

27. On February 6, 2018, Genentech supplemented its § 262(l)(3)(A) list to include a newly issued manufacturing patent: U.S. Patent No. 9,868,760.

28. On February 13, 2018, Amgen purported to provide its detailed statement concerning non-infringement and invalidity pursuant to 42 U.S.C. § 262(l)(3)(B) (“Amgen’s 3B Statement”). Amgen’s 3B Statement was deficient in numerous ways. For example, it—like Amgen’s document productions—failed to fully describe Amgen’s manufacturing process, such that Genentech was unable to evaluate many of Amgen’s non-infringement arguments.

29. On February 27, 2018, March 12, 2018, and April 13, 2018, Amgen produced additional documents regarding Amgen’s correspondence with the FDA regarding its aBLA submission. These supplemental productions still failed to fully describe Amgen’s manufacturing process.

30. On April 13, 2018, and subject to its objections, Genentech provided its response to Amgen’s 3C Statement pursuant to 42 U.S.C. § 262(l)(3)(C) (“Genentech’s 3C Statement”). Genentech included responses to Amgen’s non-infringement and invalidity statements for each of the patents addressed in Amgen’s 3B Statement and maintained that ABP 980 will infringe at least 18 Genentech patents. With its 3C Statement, Genentech proposed that Amgen agree that all 18 of these patents be included in a first-phase infringement action under § 262(l)(6).

31. On April 25, 2018, and April 30, 2018, Amgen produced additional documents regarding Amgen’s correspondence with the FDA regarding its aBLA submission. These supplemental productions still failed to fully describe Amgen’s manufacturing process.

32. After Genentech served its 3C Statement, the parties initiated negotiations under § 262(l)(4). On May 23, 2018, Genentech and Amgen agreed that the 37 patents addressed in the

exhibits to Genentech's 3C Statement shall be the subject of an action for patent infringement under § 262(l)(6).

33. In light of the parties' agreement, § 262(l)(6)(A) required Genentech to bring an action for patent infringement with respect to each of the 37 patents that were part of the parties' agreement. This action is Genentech's action pursuant to § 262(l)(6)(A).

34. On May 15, 2018, while the parties' negotiations pursuant to § 262(l)(4) were underway, Amgen purported to notify Genentech pursuant to 42 U.S.C. § 262(l)(8)(A) that it intends to commence commercial marketing of ABP 980 in the United States no earlier than 180 from May 15, 2018 (i.e., October 28, 2018).

35. On June 21, 2018, Genentech and City of Hope (collectively, "Plaintiffs") sued Amgen for infringement of all 37 patents that the parties agreed to litigate during their § 262(l)(4) negotiations. On July 19, 2018, Plaintiffs and Amgen stipulated to dismiss with prejudice all claims for infringement of U.S. Patent Nos. 6,242,177, 6,489,447, 6,586,206, 6,870,034, 7,449,184, 7,501,122, 8,044,017, 8,314,225, 8,357,301, 8,460,895, 8,691,232, 8,710,196, 8,771,988, 9,047,438, 9,080,183, 9,428,766, 9,487,809, 9,493,744, and 9,868,760 relating to ABP 980, subject to certain conditions.

36. In a letter dated November 7, 2018, pursuant to the Court's scheduling order (D.I. 44), Genentech identified to Amgen a narrowed list of 10 patents which it intended to assert against Amgen in this litigation. In the same letter, Genentech notified Amgen that it intended to assert infringement of claims 10 and 11 of U.S. App. No. 14/073,659 ("the '659 application") once issued by the U.S. Patent Office. On December 25, 2018, the '659 application issued as U.S. Patent No. 10,160,811 ("the '811 patent"). Claims 6 and 7 of the '811 patent as issued

correspond to claims 10 and 11 of the '659 application. Genentech further supplemented its § 262(l)(3)(A) list to include the '811 patent.

37. On July 23, 2019, Plaintiffs and Amgen stipulated to dismiss with prejudice all claims for infringement of U.S. Patent Nos. 6,331,415, 7,923,221, 6,407,213, 6,417,335, 9,249,218, 6,121,428, and 6,620,918 relating to ABP 980. City of Hope was dismissed as a plaintiff to this case through that stipulation. That stipulation was so-ordered by the Court on July 24, 2019. D.I. 325

38. On August 2, 2019, Genentech and Amgen stipulated to a judgment of non-infringement of U.S. Patent Nos. 8,512,983 and 9,714,293 under the Court's claim construction of "a glutamine-free production culture medium," while preserving Genentech's ability to challenge that claim construction on appeal. That stipulation was so-ordered by the Court on August 20, 2019. D.I. 340.

AMGEN'S aBLA PRODUCT

39. Amgen has publicly stated that its aBLA product is biosimilar to Herceptin[®]. For example, Amgen has issued press releases claiming that ABP 980 is "a biosimilar candidate to Herceptin[®]" and "ABP 980 is a biosimilar candidate to trastuzumab,"⁴ and it has announced the results of an Amgen study that purports to conclude that "[e]fficacy, safety and immunogenicity data support ABP 980 as a trastuzumab biosimilar."⁵

40. Given Amgen's claim of biosimilarity, Amgen's aBLA product must "utilize the same mechanism or mechanisms of action [as Herceptin[®]] for the condition or conditions of use

⁴ <http://www.amgen.com/media/news-releases/2017/07/amgen-and-allergan-submit-biosimilar-biologics-license-application-for-abp-980-to-us-food-and-drug-administration/>

⁵ <https://www.amgen.com/media/news-releases/2017/09/amgen-and-allergan-present-phase-3-data-on-biosimilar-trastuzumab-candidate-abp-980-at-the-european-society-for-medical-oncology-2017-congress/>

prescribed, recommended, or suggested in the proposed labeling.” 42 U.S.C.

§ 262(k)(2)(A)(i)(II).

41. Under 35 U.S.C. § 271(e)(2)(C), Amgen has committed a statutory act of patent infringement with respect to patents identified by Genentech under 42 U.S.C. § 262(l)(3), through the submission of its aBLA application for ABP 980.

42. On June 13, 2019, Amgen’s aBLA product was approved by the FDA.

43. On July 18, 2019, Amgen issued a press release announcing that “KANJINTI™ (trastuzumab-anns), a biosimilar to Herceptin® (trastuzumab), [is] now available in the United States (U.S.).”⁶

44. Since making its aBLA product available to customers in the United States, Amgen has stated that it “has received confirmation from its customers that they have begun administering Kanjinti® to cancer patients.”⁷

GENENTECH’S ASSERTED PATENTS

45. Genentech has spent over two decades and significant resources developing Herceptin®, and the USPTO has awarded to Genentech numerous patents on innovations resulting from this massive undertaking.

46. Upon information and belief, Amgen’s aBLA product infringes or will infringe at least the following patents, which Genentech has asserted in this lawsuit: U.S. Patent No. 7,846,441, U.S. Patent No. 7,892,549, U.S. Patent No. 6,627,196, U.S. Patent No. 7,371,379,

⁶ <http://investors.amgen.com/news-releases/news-release-details/amgen-and-allergans-mvasitm-bevacizumab-awwb-and-kanjintitm>

⁷ Second Declaration of Robert Jacobson in Support of Amgen Inc.’s Opposition to Genentech, Inc.’s Emergency Motion for an Injunction Pending Appeal, *Genentech, Inc. v. Amgen Inc.*, No. 19-2156 (Fed. Cir.), ECF No. 28 (July 29, 2019).

U.S. Patent No. 10,160,811, U.S. Patent No. 8,574,869, U.S. Patent No. 7,993,834, U.S. Patent No. 8,076,066, and U.S. Patent No. 8,440,402.

The Combination Chemotherapy Patents

47. U.S. Patent No. 7,846,441 (“the ’441 patent”), claims the administration of Herceptin® in combination with a chemotherapy agent known as a taxoid, in the absence of an anthracycline derivative (another chemotherapy agent) in an amount effective to extend time to disease progression without overall increase in severe adverse events. This specific method of treatment unexpectedly resulted in a significant improvement in patient outcomes. It nearly doubled the time until disease progression compared to treatment using a taxoid alone, and it also avoided the serious cardiotoxicity associated with Herceptin® in combination with anthracycline derivatives that unexpectedly presented during the Herceptin® clinical trials.

48. The ’441 patent, titled “Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on December 7, 2010. A true and correct copy of the ’441 patent is attached as Exhibit A. Genentech is the owner by assignment of the ’441 patent.

49. U.S. Patent No. 7,892,549 (“the ’549 patent”) is a continuation to the ’441 patent that claims a method of treating a patient with HER2-positive breast cancer by administering Herceptin® in combination with a taxoid and a further growth inhibitory agent or further therapeutic agent.

50. The ’549 patent, titled “Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on February 22, 2011. A true and correct copy of the ’549 patent is attached as Exhibit B. Genentech is the owner by assignment of the ’549 patent.

The Method of Administration Patents

51. U.S. Patent Nos. 6,627,196, 7,371,379, and 10,160,811 (collectively, the “Method of Administration Patents”) generally cover the most common administration method for Herceptin[®]: an initial dose of 8 mg/kg, followed by 6 mg/kg doses once every three weeks. Herceptin[®] was initially approved for administration on a weekly regimen, but Genentech discovered that the drug could be dosed only once every three weeks without reducing safety or effectiveness. The discovery of three-weekly dosing has had a marked impact on patients’ quality of life by providing the same life-saving effects of Herceptin[®] while allowing patients to receive treatment less frequently.

52. U.S. Patent No. 6,627,196 (“’196 patent”), titled “Dosages for Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on September 30, 2003. A true and correct copy of the ’196 patent is attached as Exhibit C. Genentech is the owner by assignment of the ’196 patent.

53. U.S. Patent No. 7,371,379 (“the ’379 patent”), titled “Dosages for Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on May 13, 2008. A true and correct copy of the ’379 patent is attached as Exhibit D. Genentech is the owner by assignment of the ’379 patent.

54. U.S. Patent No. 10,160,811 (“the ’811 patent”), titled “Treatment with Anti-ErbB2 Antibodies,” was duly and legally issued by the Patent Office on December 25, 2018. A true and correct copy of the ’811 patent is attached as Exhibit E. Genentech is the owner by assignment of the ’811 patent.

HER2 Diagnostic Patents

55. U.S. Patent Nos. 7,993,834, 8,076,066, and 8,440,402 claim novel techniques for identifying patients who might benefit from trastuzumab therapy using gene amplification techniques even where immunohistochemistry techniques suggest that the patient may not overexpress HER2.

56. U.S. Patent No. 7,993,834 (“the ’834 patent”), titled “Detection of ErbB2 Gene Amplification to Increase the Likelihood of the Effectiveness of ErbB2 Antibody Breast Cancer Therapy,” was duly and legally issued by the Patent Office on August 9, 2011. A true and correct copy of the ’834 patent is attached as Exhibit F. Genentech is the owner by assignment of the ’834 patent.

57. U.S. Patent No. 8,076,066 (“the ’066 patent”), titled “Gene Detection Assay for Improving the Likelihood of an Effective Response to a HER2 Antibody Cancer Therapy,” was duly and legally issued by the Patent Office on December 13, 2011. A true and correct copy of the ’066 patent is attached as Exhibit G. Genentech is the owner by assignment of the ’066 patent.

58. U.S. Patent No. 8,440,402 (“the ’402 patent”), titled “Gene Detection Assay for Improving the Likelihood of an Effective Response to a HER2 Antibody Cancer Therapy,” was duly and legally issued by the Patent Office on May 14, 2013. A true and correct copy of the ’402 patent is attached as Exhibit H. Genentech is the owner by assignment of the ’402 patent.

The Kao Patent

59. U.S. Patent No. 8,574,869 (“the ’869 patent”), titled “Prevention of Disulfide Bond Reduction During Recombinant Production of Polypeptides,” was duly and legally issued

by the Patent Office on November 5, 2013. A true and correct copy of the '869 patent is attached as Exhibit I. Genentech is the owner by assignment of the '869 patent.

**COUNT I
INFRINGEMENT OF U.S. PATENT NO. 7,846,441**

60. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

61. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '441 patent. Genentech included the '441 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '441 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

62. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '441 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '441 patent under 35 U.S.C. § 271(e)(2)(C)(i).

63. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '441 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement. Such

infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

64. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '441 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

65. Amgen has knowledge of and is aware of the '441 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '441 patent is willful.

66. By obtaining FDA approval of a package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '441 patent, either literally or under the doctrine of equivalents.

67. Upon information and belief, Amgen is aware, has knowledge, and/or is willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's package insert and, therefore, will directly infringe at least one claim of the '441 patent, either literally or under the doctrine of equivalents.

68. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '441 patent, either literally or under the doctrine of equivalents, by at least Amgen's package insert for the Amgen aBLA product.

69. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '441 patent.

70. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '441 patent. Genentech has no adequate remedy at law.

71. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT II
INFRINGEMENT OF U.S. PATENT NO. 7,892,549

72. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

73. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '549 patent. Genentech included the '549 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '549 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

74. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '549 patent. Amgen has

therefore committed a technical act of infringement of one or more claims of the '549 patent under 35 U.S.C. § 271(e)(2)(C)(i).

75. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '549 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

76. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '549 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

77. Amgen has knowledge of and is aware of the '549 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '549 patent is willful.

78. By obtaining FDA approval of a package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '549 patent, either literally or under the doctrine of equivalents.

79. Upon information and belief, Amgen is aware, has knowledge, and/or is willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe

and/or administer the Amgen aBLA product according to Amgen's package insert and, therefore, will directly infringe at least one claim of the '549 patent, either literally or under the doctrine of equivalents.

80. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '549 patent, either literally or under the doctrine of equivalents, by at least Amgen's package insert for the Amgen aBLA product.

81. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '549 patent.

82. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '549 patent. Genentech has no adequate remedy at law.

83. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

**COUNT III
INFRINGEMENT OF U.S. PATENT NO. 6,627,196**

84. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

85. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '196

patent. Genentech included the '196 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '196 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

86. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '196 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '196 patent under 35 U.S.C. § 271(e)(2)(C)(i).

87. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '196 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement and Genentech's infringement contentions served in this case. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

88. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '196 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

89. Amgen has knowledge of and is aware of the '196 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '196 patent is willful.

90. By obtaining FDA approval of a package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '196 patent, either literally or under the doctrine of equivalents.

91. Upon information and belief, Amgen is aware, has knowledge, and/or is willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's package insert and, therefore, will directly infringe at least one claim of the '196 patent, either literally or under the doctrine of equivalents.

92. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '196 patent, either literally or under the doctrine of equivalents, by at least Amgen's package insert for the Amgen aBLA product.

93. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '196 patent.

94. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '196 patent. Genentech has no adequate remedy at law.

95. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT IV
INFRINGEMENT OF U.S. PATENT NO. 7,371,379

96. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

97. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '379 patent. Genentech included the '379 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '379 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

98. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '379 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '379 patent under 35 U.S.C. § 271(e)(2)(C)(i).

99. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '379 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement and Genentech's infringement contentions served in this case. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

100. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '379 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

101. Amgen has knowledge of and is aware of the '379 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '379 patent is willful.

102. By obtaining FDA approval of a package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '379 patent, either literally or under the doctrine of equivalents.

103. Upon information and belief, Amgen is aware, has knowledge, and/or is willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's package insert and, therefore, will directly infringe at least one claim of the '379 patent, either literally or under the doctrine of equivalents.

104. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '379 patent, either literally or under the doctrine of equivalents, by at least Amgen's package insert for the Amgen aBLA product.

105. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '379 patent.

106. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '379 patent. Genentech has no adequate remedy at law.

107. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

**COUNT V
INFRINGEMENT OF U.S. PATENT NO. 10,160,811**

108. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

109. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '811 patent. Genentech included the '811 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) as supplemented.

110. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '811 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '811 patent under 35 U.S.C. § 271(e)(2)(C)(i).

111. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '811 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the

manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product as explained in Genentech's infringement contentions served in this case. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

112. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '811 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

113. Amgen has knowledge of and is aware of the '811 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) as supplemented and the filing of this Complaint. Amgen's infringement of the '811 patent is willful.

114. By obtaining FDA approval of a package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '811 patent, either literally or under the doctrine of equivalents.

115. Upon information and belief, Amgen is aware, has knowledge, and/or is willfully blind to the fact that patients will administer and/or use and medical practitioners will prescribe and/or administer the Amgen aBLA product according to Amgen's package insert and, therefore, will directly infringe at least one claim of the '811 patent, either literally or under the doctrine of equivalents.

116. Upon information and belief, Amgen knows or should know that it will aid and abet another's direct infringement of at least one of the claims of the '811 patent, either literally

or under the doctrine of equivalents, by at least Amgen's package insert for the Amgen aBLA product.

117. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '811 patent.

118. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '811 patent. Genentech has no adequate remedy at law.

119. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

**COUNT VI
INFRINGEMENT OF U.S. PATENT NO. 7,993,834**

120. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

121. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '834 patent. Genentech included the '834 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '834 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

122. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '834 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '834 patent under 35 U.S.C. § 271(e)(2)(C)(i).

123. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '834 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement and Genentech's infringement contentions served in this case. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

124. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '834 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

125. Amgen has knowledge of and is aware of the '834 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '834 patent is willful.

126. By obtaining FDA approval of a package insert having directions that instruct patients to administer and/or use and medical practitioners to prescribe and/or administer the Amgen aBLA product, Amgen has an affirmative intent to actively induce infringement by others of one or more claims of the '834 patent, either literally or under the doctrine of

equivalents.

127. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '834 patent.

128. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '834 patent. Genentech has no adequate remedy at law.

129. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

**COUNT VII
INFRINGEMENT OF U.S. PATENT NO. 8,076,066**

130. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

131. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '066 patent. Genentech included the '066 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '066 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

132. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '066 patent. Amgen has

therefore committed a technical act of infringement of one or more claims of the '066 patent under 35 U.S.C. § 271(e)(2)(C)(i).

133. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '066 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement and Genentech's infringement contentions served in this case. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

134. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '066 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

135. Amgen has knowledge of and is aware of the '066 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '066 patent is willful.

136. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '066 patent.

137. Genentech will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '066 patent. Genentech has no adequate remedy at law.

138. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

**COUNT VIII
INFRINGEMENT OF U.S. PATENT NO. 8,440,402**

139. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

140. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '402 patent. Genentech included the '402 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '402 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

141. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '402 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '402 patent under 35 U.S.C. § 271(e)(2)(C)(i).

142. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '402 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug

substance and its ABP 980 drug product, as explained in Genentech's 3C Statement. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

143. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '402 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

144. Amgen has knowledge of and is aware of the '402 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '402 patent is willful.

145. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '402 patent.

146. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '402 patent. Genentech has no adequate remedy at law.

147. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

COUNT IX
INFRINGEMENT OF U.S. PATENT NO. 8,574,869

148. Genentech incorporates by reference paragraphs 1-59 as if fully set forth herein.

149. Upon review of publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Genentech believes that a claim of patent

infringement, either literally or under the doctrine of equivalents, could reasonably be asserted by Genentech if a person not licensed by Genentech engaged in the making, using, offering to sell, selling, or importing into the United States of ABP 980 prior to the expiration of the '869 patent. Genentech included the '869 patent in its disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A). Genentech also provided Amgen with a detailed statement that describes, on a claim-by-claim basis, the factual and legal basis of its opinion that the '869 patent will be infringed by the commercial marketing of ABP 980, pursuant to 42 U.S.C. § 262(l)(3)(C).

150. Amgen submitted its aBLA to obtain approval to engage in the commercial manufacture, use, or sale of ABP 980 before the expiration of the '869 patent. Amgen has therefore committed a technical act of infringement of one or more claims of the '869 patent under 35 U.S.C. § 271(e)(2)(C)(i).

151. Likewise, based on publicly available information and/or information provided by Amgen pursuant to 42 U.S.C. § 262(l)(2), Amgen has infringed or will infringe the '869 patent in violation of 35 U.S.C. §§ 271(a), (b), and/or (g) as a result of its activities relating to the manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and its ABP 980 drug product, as explained in Genentech's 3C Statement and Genentech's infringement contentions served in this case. Such infringement either has occurred or is imminent because, among other things, Amgen has announced that its aBLA product is now available in the United States.

152. Pursuant to 42 U.S.C. § 262(l)(9)(A) and 28 U.S.C. § 2201, Genentech is entitled to a declaratory judgment that Amgen's manufacture, importation, sale, offer for sale, use, and promotion of the use of the ABP 980 drug substance and Amgen's ABP 980 drug product has infringed or will infringe the '869 patent pursuant to 35 U.S.C. §§ 271(a), (b), and/or (g).

153. Amgen has knowledge of and is aware of the '869 patent, including due to Genentech's disclosure of patents pursuant to 42 U.S.C. § 262(l)(3)(A) and the filing of this Complaint. Amgen's infringement of the '869 patent is willful.

154. Pursuant to 35 U.S.C. § 284, Genentech is entitled to damages, including lost profits and/or a reasonable royalty, for Amgen's infringement of the '869 patent.

155. Genentech has suffered or will suffer irreparable injury for which damages are an inadequate remedy unless Amgen is enjoined from infringing the claims of the '869 patent. Genentech has no adequate remedy at law.

156. Genentech is entitled to an injunction pursuant to at least 35 U.S.C. § 271(e)(4)(B), 42 U.S.C. § 262(l)(8)(B), 35 U.S.C. § 271(a), (b), (g), and/or 35 U.S.C. § 283 preventing Amgen from the commercial manufacture, use, offer to sell, or sale within the United States of the Amgen aBLA product.

PRAYER FOR RELIEF

WHEREFORE, Genentech respectfully requests that this Court enter judgment in their favor against Amgen and grant the following relief:

- a. a judgment that Amgen has infringed or induced infringement of one or more claims of the asserted patents under 35 U.S.C. § 271(e)(2)(C);
- b. a judgment that Amgen has infringed or will infringe, or has induced or will induce infringement, of one or more claims of the asserted patents by engaging in the manufacture, import, offer for sale, sale, or use within the United States of the Amgen aBLA product before the expirations of the asserted patents under 35 U.S.C. § 271(a), (b), and/or (g);
- c. preliminary and/or permanent equitable relief, including but not limited to a preliminary and permanent injunction that enjoins Amgen, its officers, partners, agents, servants,

employees, parents, subsidiaries, affiliate corporations, other related business entities, and all other persons acting in concert, participation, or in privity with Amgen and/or its successors or assigns from infringing the asserted patents, or contributing to or inducing anyone to do the same, by acts including the manufacture, use, offer to sell, sale, distribution, or importation of any current or future versions of a product that infringes, or the use or manufacturing of which infringes the asserted patents;

d. monetary damages, including lost profits and/or a reasonable royalty, and an accounting and/or ongoing royalty for any post-judgment infringement;

e. a judgment that Amgen's infringement was willful and enhancement of any monetary damages pursuant to 35 U.S.C. § 284;

f. a declaration that this is an exceptional case and an award to Plaintiffs of their attorneys' fees, costs, and expenses pursuant to 35 U.S.C. § 271(e)(4) and 35 U.S.C. § 285; and

g. such other relief as this Court may deem just and proper.

JURY DEMAND

Plaintiff Genentech, Inc., by and through its undersigned counsel, hereby demand, pursuant to Federal Rule of Civil Procedure 38, a trial by jury on all claims so triable in this action.

Dated: August 27, 2019

Respectfully submitted,

Of Counsel:

William F. Lee
Lisa J. Pirozzolo
Emily R. Whelan
Kevin S. Prussia
Andrew J. Danford
WILMER CUTLER PICKERING
HALE AND DORR LLP
60 State Street
Boston, MA 02109
william.lee@wilmerhale.com
lisa.pirozzolo@wilmerhale.com
emily.whelan@wilmerhale.com
kevin.prussia@wilmerhale.com
andrew.danford@wilmerhale.com

Robert J. Gunther Jr.
WILMER CUTLER PICKERING
HALE AND DORR LLP
7 World Trade Center
250 Greenwich Street
New York, NY 10007
robert.gunther@wilmerhale.com

Daralyn J. Durie
Adam R. Brausa
DURIE TANGRI LLP
217 Leidesdorff St.
San Francisco, CA 94111
ddurie@durietangri.com
abrausa@durietangri.com

/s/ Daniel M. Silver

Michael P. Kelly (#2295)
Daniel M. Silver (#4758)
Alexandra M. Joyce (#6423)
MCCARTER & ENGLISH, LLP
Renaissance Centre
405 North King Street, 8th Floor
Wilmington, DE 19801
(302) 984-6300
mkelly@mccarter.com
dsilver@mccarter.com
ajoyce@mccarter.com

Counsel for Plaintiff Genentech, Inc.

EXHIBIT A

US007846441B1

(12) **United States Patent
Hellmann**(10) **Patent No.:** **US 7,846,441 B1**
(45) **Date of Patent:** **Dec. 7, 2010**

- (54) **TREATMENT WITH ANTI-ERBB2
ANTIBODIES**
- (75) Inventor: **Susan D. Hellmann**, San Carlos, CA
(US)
- (73) Assignee: **Genentech, Inc.**, South San Francisco,
CA (US)
- (*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.
- (21) Appl. No.: **09/208,649**
- (22) Filed: **Dec. 10, 1998**

Related U.S. Application Data

- (60) Provisional application No. 60/069,346, filed on Dec.
12, 1997.
- (51) **Int. Cl.**
A61K 39/395 (2006.01)
- (52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1;
424/135.1; 424/136.1; 424/141.1; 424/142.1;
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424/174.1
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Primary Examiner—Alana M. Harris
Assistant Examiner—Anne L. Holleran
 (74) *Attorney, Agent, or Firm*—Ginger R. Dreger; Atulya R.
 Agarwal

(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

14 Claims, 2 Drawing Sheets

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US 7,846,441 B1

Page 6

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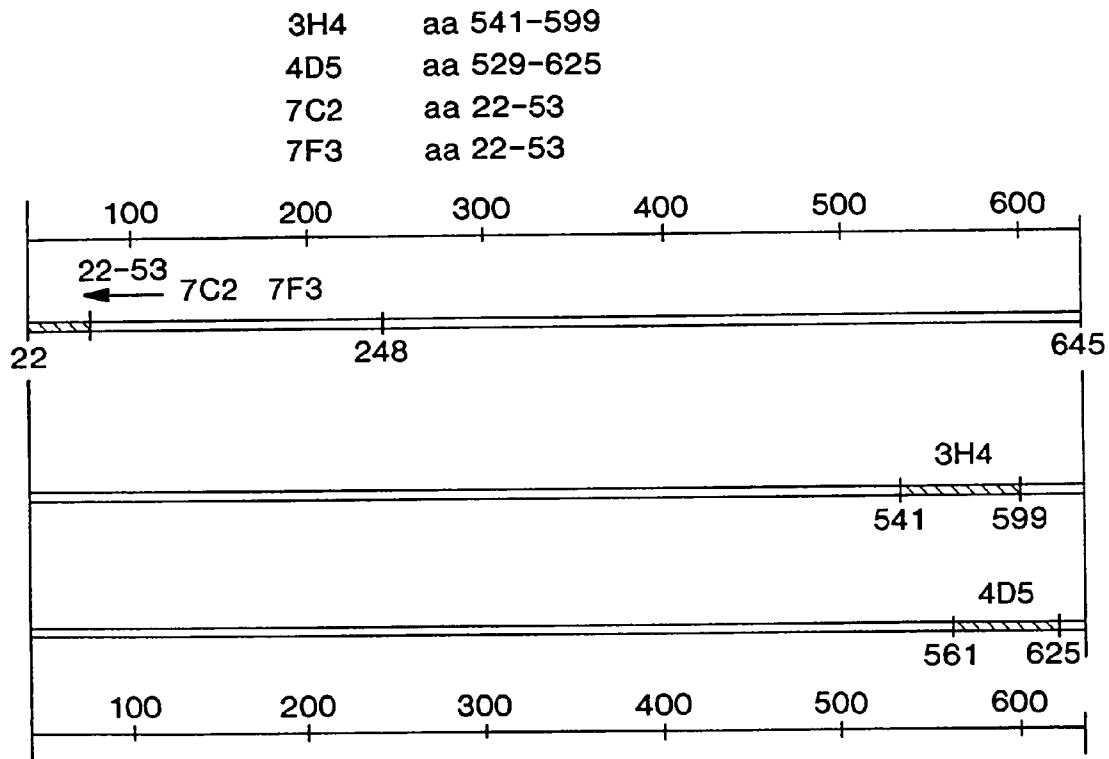
U.S. Patent

Dec. 7, 2010

Sheet 1 of 2

US 7,846,441 B1

FIG. 1



3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPFPCVAR
| |
541 599

4D5 epitope (SEQ ID NO:9) (64 residues)

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPFPCVARCPGKVPDLSYMPIWKFPDEEGACQP
| |
561 625

U.S. Patent

Dec. 7, 2010

Sheet 2 of 2

US 7,846,441 B1

1 MELAALCRWGLLLALLPPGAASTQVCTGTDMLRLPA
38 SPETHLDMLRHLYQGCVOVVOGNLELTYLPTNASLSFL
75 ODIOEVOGYVLI AHNOVROVPLORLRIVRGTOLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELOLRSLTEI
149 LKGGVLIQORNPOLCYODTILWKDIFHKNNOLALTID
186 TNRSRA

FIG. 2

US 7,846,441 B1

1

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This is a non-provisional application claiming priority to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosure of which is hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor (p185^{HER2}) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237: 178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS (USA)* 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

2

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p185^{erbB2}-overexpressing breast tumor cell lines to the cytotoxic effects of TNF- α . See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS (USA)* 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

US 7,846,441 B1

3

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMab HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dys-

4

function that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MABs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 µCi each of ³⁵S methionine and ³⁵S cysteine. Supernatants were harvested either the ErbB2 MABs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and

US 7,846,441 B1

5

electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. 2 depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the “7C2/7F3 epitope” (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms “HER2”, “ErbB2” “c-Erb-B2” are used interchangeably. Unless indicated otherwise, the terms “ErbB2” “c-Erb-B2” and “HER2” when used herein refer to the human protein and “her2”, “erbB2” and “c-erb-B2” refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al, *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The “epitope 4D5” is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. 1) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The “epitope 3H4” is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. 1, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The “epitope 7C2/7F3” is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term “induces cell death” or “capable of inducing cell death” refers to the ability of the antibody to make a viable cell become nonviable. The “cell” here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which “overexpresses” ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity

6

(CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the “PI uptake assay in BT474 cells”.

The phrase “induces apoptosis” or “capable of inducing apoptosis” refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the “cell” is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an “annexin binding assay using BT474 cells” (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

“Heregulin” (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3):1909-1919 (1994); and Marchionni et al., *Nature*, 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. HRGβ1₁₇₇₋₂₄₄).

The “ErbB2-ErbB3 protein complex” and “ErbB2-ErbB4 protein complex” are noncovalently associated oligomers of

US 7,846,441 B1

7

the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20): 14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No. 91-3242*, Vol. I, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs

8

specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the

US 7,846,441 B1

9

techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include “chimeric” antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al, *Proc. Natl. Acad. Sci. USA*, 81; 6851-6855 [1984]).

“Humanized” forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al, *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

“Single-chain Fv” or “sFv” antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term “diabodies” refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

10

An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term “salvage receptor binding epitope” refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

“Treatment” refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

“Mammal” for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A “disorder” is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term “therapeutically effective amount” is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term “cytotoxic agent” as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I¹³¹, I¹²⁵, Y⁹⁰ and Re¹⁸⁶), chemo-

US 7,846,441 B1

11

therapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside (“Ara-C”), cyclophosphamide, thiotepea, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (Taxotere®, Rhône-Poulenc Rorer, Antony, France), methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, caminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A “growth inhibitory agent” when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled “Cell cycle regulation, oncogenes, and antineoplastic drugs” by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

“Doxorubicin” is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term “cytokine” is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and - β ; mul-
lerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- β ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 α , IL-2,

12

IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF- α or TNF- β ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term “prodrug” as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, “Prodrugs in Cancer Chemotherapy” *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., “Prodrugs: A Chemical Approach to Targeted Drug Delivery,” *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxycetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By “solid phase” is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A “liposome” is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS (USA)* 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

US 7,846,441 B1

13

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with $\frac{1}{5}$ to $\frac{1}{10}$ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego,

14

Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130: 151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348: 552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc.*

US 7,846,441 B1

15

Natl. Acad. Sci. USA, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

16

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

US 7,846,441 B1

17

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe

18

a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')₂ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5): 1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties
Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10⁶ per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the appropriate

US 7,846,441 B1

19

MAB. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca²⁺ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and aliquoted into 35 mm strainer-capped 12x75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an “annexin binding assay using BT474 cells” is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the MAB. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca²⁺ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FITC) (1 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a “DNA staining assay using BT474 cells” is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 µg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin/streptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 µg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased

20

complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced antitumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y and ¹⁸⁶Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepen-taetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a “receptor” (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a “ligand” (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-de-

US 7,846,441 B1

21

rivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19):1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

22

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNS-SMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or F(ab')₂. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNS-SMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonyl fluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography.

US 7,846,441 B1

23

phy, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition,

24

the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, e.g. doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

US 7,846,441 B1

25

The treatment of the present invention involved the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Chemotherapy Service Ed.*, M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may

26

have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package insert with instructions for use, including a warning that the composition is not to be used in combination with anthracycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Va. 20110-2209 (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12226	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

Example

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 IgG₁κ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3₄₀₀ cells (expressing approximately 1×10⁵ ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci. (USA)* 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10⁷ cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ³²P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG₁ (IgG₁) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185" (Dilohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of p185^{HER2}, induces antibody-dependent

US 7,846,441 B1

27

cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to over-express ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p185^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs.

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women ≥ 18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer
Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

28

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m²) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m²) or epirubicin (75 mg/m²) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paclitaxel (TAXOL®) was given at a dose of 175 mg/m² over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H₂ blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

US 7,846,441 B1

29

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68

30

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	Enrolled	TTP(months)	RR(%)	AE(%)
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p <0.001 by log-rank test
**p <0.01 by X² test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
50 55 60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
65 70 75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
80 85 90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
95 100 105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
110 115 120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
125 130 135

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys
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Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg
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US 7,846,441 B1

31

32

-continued

Ala
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US 7,846,441 B1

33

34

-continued

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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
 35 40 45

Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
 50 55 60

Gly Ala Cys Gln Pro
 65

The invention claimed is:

1. A method for the treatment of a human patient with a malignant progressing tumor or cancer characterized by over-expression of ErbB2 receptor, comprising administering a combination of an intact antibody which binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

2. The method of claim 1 wherein said patient has a malignant tumor.

3. The method of claim 1 wherein said patient has cancer.

4. The method of claim 3 wherein said cancer is selected from the group consisting of breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

5. The method of claim 4 wherein said cancer is breast cancer.

6. The method of claim 5 wherein said cancer is metastatic breast carcinoma.

7. The method of claim 1 wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

8. The method of claim 1 wherein said taxoid is paclitaxel.

9. The method of claim 8 wherein the effective amount of said combination is lower than the sum of the effective amounts of said anti-ErbB2 antibody and said taxoid, when administered individually, as single agents.

10. The method of claim 1 wherein efficacy is further measured by determining the response rate.

11. A method for the treatment of a human patient with ErbB2 overexpressing progressing metastatic breast cancer, comprising administering a combination of a humanized 4D5 anti-ErbB2 antibody and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

12. The method of claim 11 wherein said taxoid is paclitaxel.

US 7,846,441 B1

35

13. A method for the treatment of a human patient with a progressing malignant tumor or cancer characterized by over-expression of ErbB2 receptor, comprising administering a combination of a humanized 4D5 anti-ErbB2 antibody which comprises a human Fc region and that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

36

14. A method for the treatment of a human patient with ErbB2 expressing progressing metastatic breast cancer, comprising administering a combination of an antibody which binds to epitope 4D5 within the extracellular domain sequence and a taxoid, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in said human patient, without increase in overall severe adverse events.

* * * * *

EXHIBIT B

US007892549B2

(12) **United States Patent**
Paton et al.(10) **Patent No.:** **US 7,892,549 B2**
(45) **Date of Patent:** ***Feb. 22, 2011**

- (54) **TREATMENT WITH ANTI-ERBB2 ANTIBODIES**
- (75) Inventors: **Virginia E. Paton**, Oakland, CA (US);
Steven Shak, Burlingame, CA (US);
Susan D. Hellmann, San Carlos, CA (US)
- (73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)
- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1827 days.
- This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/356,824**(22) Filed: **Feb. 3, 2003**(65) **Prior Publication Data**

US 2004/0037823 A9 Feb. 26, 2004

Related U.S. Application Data

- (63) Continuation of application No. 09/208,649, filed on Dec. 10, 1998.
- (60) Provisional application No. 60/069,346, filed on Dec. 12, 1997.

(51) **Int. Cl.**

A61K 39/395 (2006.01)
C07K 16/28 (2006.01)
C07K 16/30 (2006.01)

- (52) **U.S. Cl.** **424/143.1**; 424/130.1; 424/133.1;
424/134.1; 424/135.1; 424/136.1; 424/138.1;
424/141.1; 424/152.1; 424/155.1; 424/156.1;
424/172.1; 424/174.1

- (58) **Field of Classification Search** 424/130.1,
424/133.1, 138.1, 141.1, 143.1, 155.1, 174.1,
424/134.1, 135.1, 136.1, 152.1, 156.1, 172.1
See application file for complete search history.

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Primary Examiner—Alana M. Harris
Assistant Examiner—Anne L. Holleran
(74) *Attorney, Agent, or Firm*—Arnold & Porter LLP; Diane Marschang; Ginger R. Dreger

(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

US 7,892,549 B2

Page 2

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US 7,892,549 B2

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Feb. 22, 2011

US 7,892,549 B2

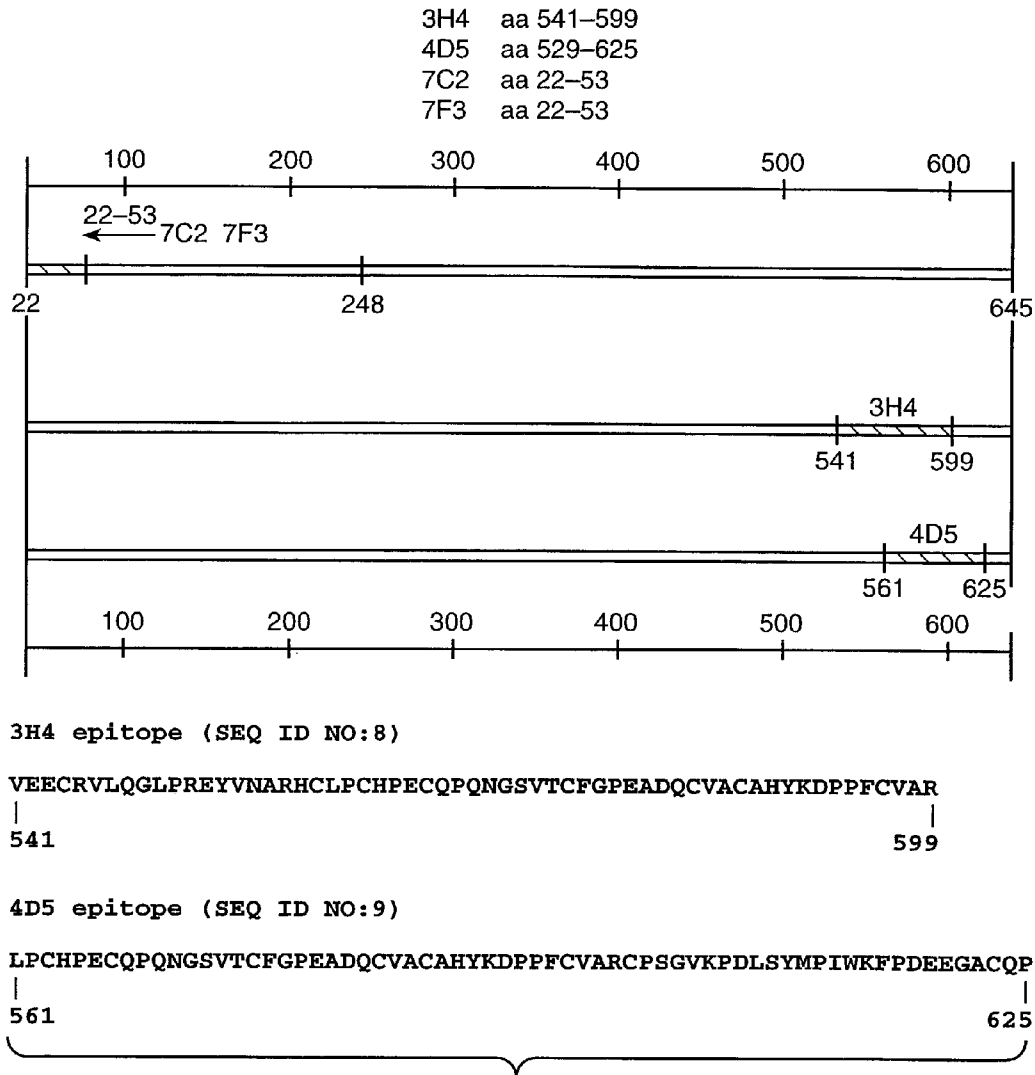


FIG._1

1 MELAALCRWGLLLALLPPGAASTQVCTGTDMLRLPA
38 SPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG._2

US 7,892,549 B2

1

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

This is a continuation of non-provisional application Ser. No. 09/208,649, filed Dec. 10, 1998, which claims priority under 35 USC §119 to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosures of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor (p185^{HER2}) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712[1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:178-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B 104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al *PNAS* (USA) 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects

2

of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p185^{erbB2}-overexpressing breast tumor cell lines to the cytotoxic effects of TNF- α . See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369[1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS* (USA) 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of

US 7,892,549 B2

3

this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2 or HERCEPTIN®) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]).

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198: 165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 2):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMAb HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the treatment of disorders characterized by overexpression of ErbB2, and is based on the recognition that while treatment with anti-ErbB2 antibodies

4

markedly enhances the clinical benefit of the use of chemotherapeutic agents in general, a syndrome of myocardial dysfunction that has been observed as a side-effect of anthracycline derivatives is increased by the administration of anti-ErbB2 antibodies.

Accordingly, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of a combination of an anti-ErbB2 antibody and a chemotherapeutic agent other than an anthracycline derivative, e.g. doxorubicin or epirubicin, in the absence of an anthracycline derivative, to the human patient.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Although an antiproliferative effect is sufficient, in a preferred embodiment, the anti-ErbB2 antibody is capable of inducing cell death or is capable of inducing apoptosis. Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAb 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μ Ci each of 35 S methionine and 35 S cysteine. Supernatants were harvested either the ErbB2 MABs or control antibodies were added to the supernatant

US 7,892,549 B2

5

and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. 2 depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the “7C2/7F3 epitope” (SEQ ID NO:2).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms “HER2”, “ErbB2”, “c-Erb-B2” are used interchangeably. Unless indicated otherwise, the terms “ErbB2” “c-Erb-B2” and “HER2” when used herein refer to the human protein and “her2”, “erbB2” and “c-erb-B2” refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS* (USA) 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The “epitope 4D5” is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. 1) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The “epitope 3H4” is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. 1, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The “epitope 7C2/7F3” is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term “induces cell death” or “capable of inducing cell death” refers to the ability of the antibody to make a viable cell become nonviable. The “cell” here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which “overexpresses” ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distin-

6

guish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the “PI uptake assay in BT474 cells”.

The phrase “induces apoptosis” or “capable of inducing apoptosis” refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the “cell” is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an “annexin binding assay using BT474 cells” (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

“Heregulin” (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3):1909-1919 (1994); and Marchionni et al., *Nature*, 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. HRGβ1₁₇₇₋₂₄₄).

US 7,892,549 B2

7

The “ErbB2-ErbB3 protein complex” and “ErbB2-ErbB4 protein complex” are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20): 14661-14665 (1994).

“Antibodies” (Abs) and “immunoglobulins” (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

“Native antibodies” and “native immunoglobulins” are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term “variable” refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. 1, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

“Fv” is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer antigen-

8

binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The “light chains” of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term “antibody” is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

“Antibody fragments” comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al. *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The “monoclonal antibodies”

US 7,892,549 B2

9

may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

10

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I¹³¹, I¹²⁵, Y⁹⁰ and Re¹⁸⁶), chemo-

US 7,892,549 B2

11

therapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside (“Ara-C”), cyclophosphamide, thiotepea, busulfan, cytoxin, taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France), methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A “growth inhibitory agent” when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled “Cell cycle regulation, oncogenes, and antineoplastic drugs” by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

“Doxorubicin” is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term “cytokine” is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and - β ; mul-
lerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- β ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1a, IL-2,

12

IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF- α or TNF- β ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term “prodrug” as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, “Prodrugs in Cancer Chemotherapy” *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., “Prodrugs: A Chemical Approach to Targeted Drug Delivery,” *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxycetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By “solid phase” is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A “liposome” is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al. *PNAS* (USA) 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

US 7,892,549 B2

13

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}_1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with $\frac{1}{5}$ to $\frac{1}{10}$ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego,

14

Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Pluckthun, *Immunol. Revs.*, 130: 151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348: 552-554(1990). Clackson et al., *Nature*, 352:624-628(1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc.*

US 7,892,549 B2

15

Natl Acad. Sci. USA, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "bestfit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

16

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

US 7,892,549 B2

17

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229:81 (1985) describe a

18

procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175:217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')₂ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147:60 (1991).

(vi) Screening for Antibodies with the Desired Properties
Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10⁶ per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the appropriate

US 7,892,549 B2

19

MAB. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca²⁺ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and aliquoted into 35 mm strainer-capped 12x75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an “annexin binding assay using BT474 cells” is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the MAB. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca²⁺ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FITC) (1 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a “DNA staining assay using BT474 cells” is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 µg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin/streptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 µg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased

20

complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced antitumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcumin, croton, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y and ¹⁸⁶Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentacetic acid (MXDTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a “receptor” (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a “ligand” (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-de-

US 7,892,549 B2

21

rivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. *Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. *J. National Cancer Inst.* 81(19):1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO. 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328:457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312:604-608 [1984]).

(xi) Antibody-salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

22

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNS-SMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNISDGGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or F(ab')₂. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNS-SMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonyl fluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography.

US 7,892,549 B2

23

phy, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABXTM resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSETM chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICSTM or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition,

24

the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, e.g. doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

IV. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

US 7,892,549 B2

25

The treatment of the present invention involved the combined administration of an anti-ErbB2 antibody and a chemotherapeutic agent, other than an anthracycline derivative. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Chemotherapy Service Ed.*, M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. In a preferred embodiment, the ErbB2 antibody is co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Further information about suitable dosages is provided in the Example below.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may

26

have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture comprises a package insert with instructions for use, including a warning that the composition is not to be used in combination with anthracycline-type chemotherapeutic agent, e.g. doxorubicin, or epirubicin.

DEPOSIT OF MATERIALS

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Example.

EXAMPLE

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 IgG₁K murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3HER2-3₄₀₀ cells (expressing approximately 1×10⁵ ErbB2 molecules/cell) produced as described in Hudziak et al. *Proc. Natl. Acad. Sci. (USA)* 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10⁷ cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ³²P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN®). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG₁ (IgG₁) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185^{HER2} (Dilohiation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that con-

US 7,892,549 B2

27

tain high levels of p185^{HER2}, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to over-express ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p185^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women ≥ 18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer
Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+HER2 overexpression, or the metastatic site must have 2+ to 3+HER2 overexpression)

28

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m²) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m²) or epirubicin (75 mg/m²) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paclitaxel (TAXOL®) was given at a dose of 175 mg/m² over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mgx2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H2 blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

US 7,892,549 B2

29

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed.2), New York, N.Y., Wiley, 1981, pp 13-17).

RESULTS

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN®, without increase in overall severe adverse events (AE):

	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68

30

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	Enrolled	TTP(months)	RR(%)	AE(%)
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test
**p < 0.001 by X² test
CRx: chemotherapy
AC: anthracycline/cyclophosphamide treatment
H: HERCEPTIN ®
T: TAXOL ®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor the combined treatment with HERCEPTIN® and paclitaxel (TAXOL®).

The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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35 40 45

Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
50 55 60

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
65 70 75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
80 85 90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
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Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
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Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
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Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys
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US 7,892,549 B2

31

32

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US 7,892,549 B2

33

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Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
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Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
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Gly Ala Cys Gln Pro
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The invention claimed is:

1. A method for the treatment of a human patient with breast cancer that overexpresses ErbB2 receptor, comprising administering a combination of an antibody that binds ErbB2, a taxoid, and a further growth inhibitory agent to the human patient in an amount effective to extend the time to disease progression in the human patient, wherein the antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

2. The method of claim 1 wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

3. The method of claim 1 wherein the antibody cross-blocks binding of 4D5 to the ErbB2 extracellular domain sequence.

4. The method of claim 1 wherein the antibody binds to amino acid residues in the region from about residue 529 to about residue 625 of the ErbB2 extracellular domain sequence.

5. A method for the treatment of a human patient with breast cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody which binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid, and a further therapeutic agent, to the human patient.

6. The method of claim 5 wherein the breast cancer is metastatic breast carcinoma.

7. The method of claim 5 wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

8. The method of claim 7 wherein the antibody is administered as a 4 mg/kg dose and then weekly administration of 2 mg/kg.

9. The method of claim 5 wherein the taxoid is paclitaxel.

10. The method of claim 5 wherein efficacy is measured by determining the time to disease progression or the response rate.

11. The method of claim 5, wherein the further therapeutic agent is selected from the group consisting of: another ErbB2 antibody, EGFR antibody, ErbB3 antibody, ErbB4 antibody, vascular endothelial growth factor (VEGF) antibody, cytokine, and growth inhibitory agent.

12. The method of claim 5 wherein the further therapeutic agent is another ErbB2 antibody.

13. The method of claim 5 wherein the further therapeutic agent is a vascular endothelial growth factor (VEGF) antibody.

14. The method of claim 5 wherein the further therapeutic agent is a growth inhibitory agent.

15. The method of claim 14 wherein the growth inhibitory agent is a DNA alkylating agent.

16. A method for the treatment of a human patient with ErbB2 overexpressing breast cancer, comprising administering a combination of an antibody that binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in the human patient.

17. The method of claim 16 wherein the breast cancer is metastatic breast carcinoma.

* * * * *

EXHIBIT C



US006627196B1

(12) **United States Patent**
Baughman et al.

(10) **Patent No.: US 6,627,196 B1**
(45) **Date of Patent: Sep. 30, 2003**

- (54) **DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES**
- (75) Inventors: **Sharon A. Baughman**, Ventura, CA (US); **Steven Shak**, Burlingame, CA (US)
- (73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)
- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.
- (21) Appl. No.: **09/648,067**
- (22) Filed: **Aug. 25, 2000**

Related U.S. Application Data

- (60) Provisional application No. 60/213,822, filed on Jun. 23, 2000, and provisional application No. 60/151,018, filed on Aug. 27, 1999.
- (51) **Int. Cl.⁷** **A61K 39/395**
- (52) **U.S. Cl.** **424/138.1; 424/131.1; 424/133.1; 424/134.1; 424/135.1; 424/136.1; 424/137.1; 424/139.1; 424/141.1; 424/142.1; 424/143.1; 424/144.1; 424/145.1; 424/146.1; 424/147.1; 424/150.1; 424/151.1; 424/152.1; 424/153.1; 424/154.1; 424/155.1; 424/156.1; 424/158.1; 424/172.1; 424/174.1**
- (58) **Field of Search** 424/130.1, 138.1, 424/141.1, 142.1, 152.1, 155.1, 131.1, 133.1, 134.1, 135.1, 136.1, 137.1, 139.1, 143.1, 144.1, 145.1, 146.1, 147.1, 150.1, 151.1, 153.1, 154.1, 156.1, 158.1, 172.1, 174.1

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Primary Examiner—Anthony C. Caputa
Assistant Examiner—Anne L. Holleran
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 with anti-ErbB2 antibody.

33 Claims, 5 Drawing Sheets

US 6,627,196 B1

Page 2

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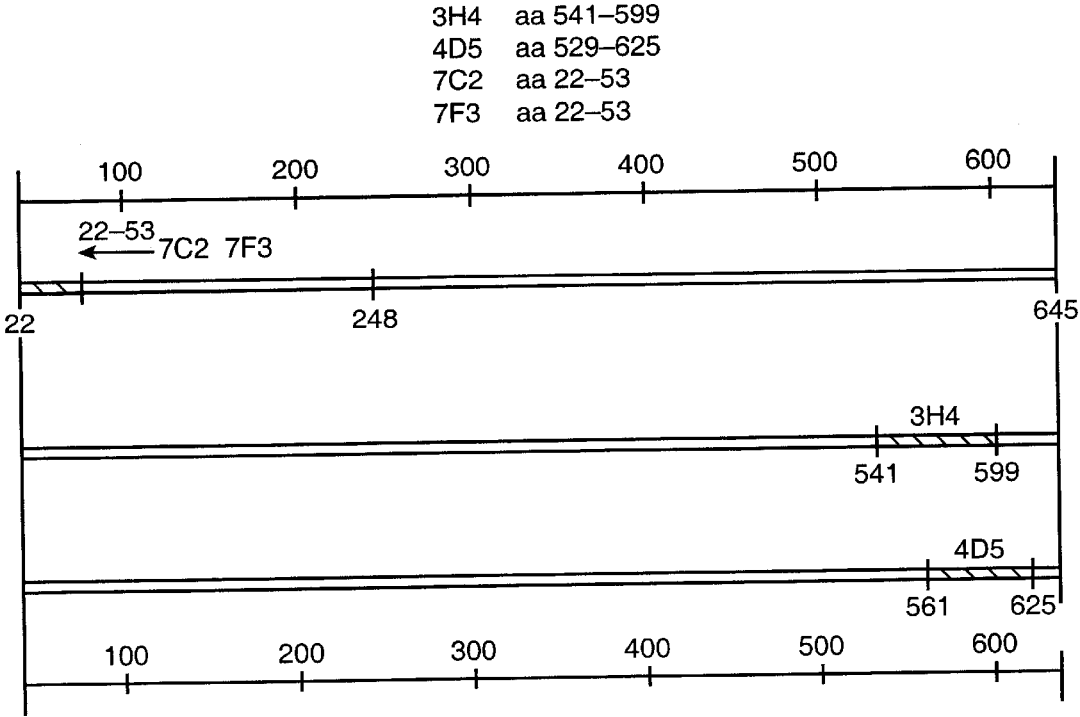
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3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR

541599

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQP

561625

FIG._1

1

MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPA

38

SPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFL

75

QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN

112

YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI

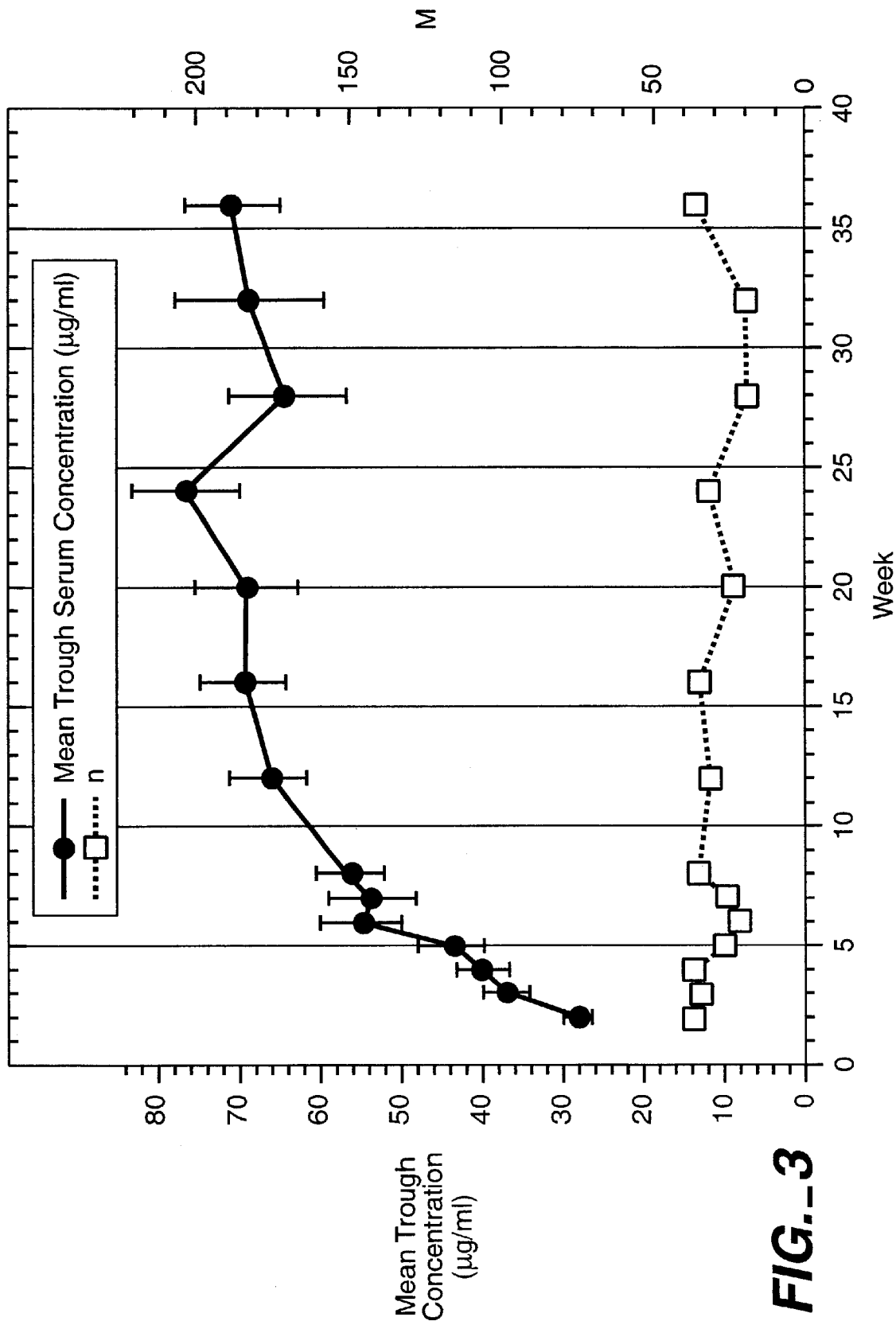
149

LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID

186

TNRSRA

FIG._2



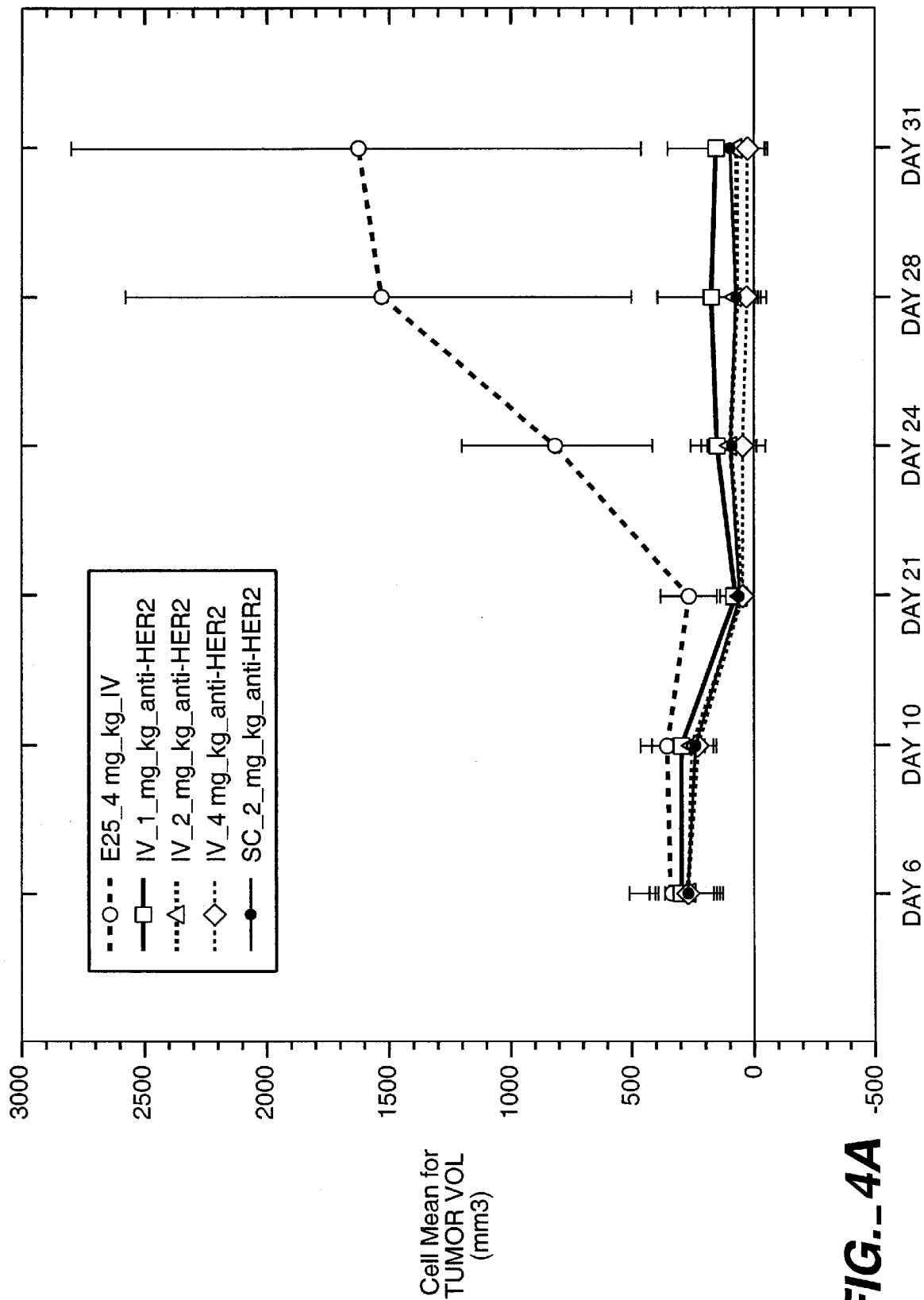


FIG. 4A

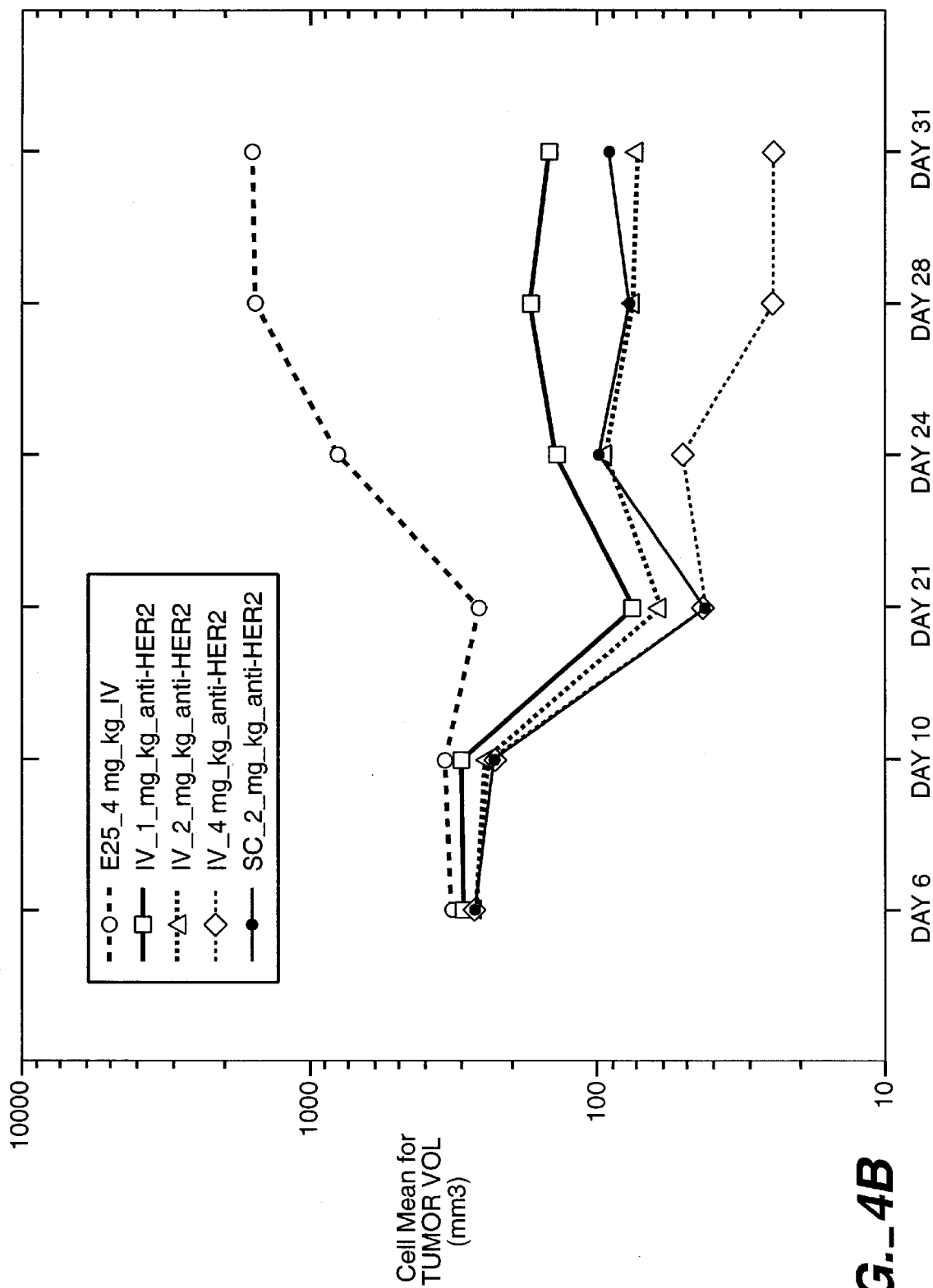


FIG. 4B

VARIABLE LIGHT

	1	10	20	30	40
2C4	DTVMTQSHKIMSTSVGDRVSITC	[KASQDVSIGVA----	WYQQR	P	
	**	**** *	*		*
574	DIQMTQSPSSLSASVGDRVTITC	[KASQDVSIGVA----	WYQQK	P	
			* * ****		
hum kI	DIQMTQSPSSLSASVGDRVTITC	[RASQSVSTSSYSYMH]	WYQQK	P	
		50	60	70	80
2C4	GQSPKLLIY	[SASYRYT]	GVPDRFTGSGSGTDFTFTISSVQA		
	**		* *	* *	*
574	GKAPKLLIY	[SASYRYT]	GVPSRFSGSGSGTDFTLTISLQP		
		*	****		
hum kI	GKAPKLLIY	[AASSLES]	GVPSRFSGSGSGTDFTLTISLQP		
		90	100		
2C4	EDLAVYYC	[QQYYIYPYT]	FGGGTKLEIK	(SEQ ID NO:10)	
	* *		* *		
574	EDFATYYC	[QQYYIYPYT]	FGQGTKVEIK	(SEQ ID NO:12)	

hum kI	EDFATYYC	[QQYNSLPYT]	FGQGTKVEIK	(SEQ ID NO:14)	

FIG._5A

VARIABLE HEAVY

	1	10	20	30	40
2C4	EVQLQQSGPELVKPGTSVKISCKAS	[GFTFTDYTMD]	WVKQS		
	**	** * * ****	**		*
574	EVQLVESGGGLVQPGGSLRLSCAAS	[GFTFTDYTMD]	WVRQA		
			** * *		
humIII	EVQLVESGGGSVQPGGSLRLSCAAS	[GFTFSSYAMS]	WVRQA		
		50	60	70	80
2C4	HGKSLEWIG	[DVNPNSGGSIYNQRFKG]	KASLTVDRSSRIVYM		
	* *	**		*** *	**** *
574	PGKGLEWVA	[DVNPNSGGSIYNQRFKG]	RFTLSVDRSKNTLYL		
		* *****	** *****	* * *	
humIII	PGKGLEWVS	[VISGDGGSTYYADSVKG]	RFTISRDDSKNTLYL		
		90	100	110	
2C4	ELRSLTFEDTAVYYCAR	[NLGPSFYFDY]	WGQGTTLVTSS	(SEQ ID NO:11)	
	***	**		*	
574	QMNSLRAEDTAVYYCAR	[NLGPSFYFDY]	WGQGTTLVTVSS	(SEQ ID NO:13)	
		** ****			
humIII	QMNSLRAEDTAVYYCAR	[GRGGGS--DY]	WGQGTTLVTVSS	(SEQ ID NO:15)	

FIG._5B

US 6,627,196 B1

1

DOSAGES FOR TREATMENT WITH ANTI-ERBB2 ANTIBODIES

RELATED APPLICATIONS

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application No. 60/151,018, filed Aug. 27, 1999 and No. 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor (p185^{HER2}) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:78-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS (USA)* 83:9129-9133 (1986), the 7.16.4 antibody was shown to

2

inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994.

Hudziak et al., *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p 185-overexpressing breast tumor cell lines to the cytotoxic effects of TNF- α . See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20): 14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS (USA)* 88:8691–8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580–2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress-ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401–408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771–2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575–4580 (1991); Shawver et al. *Cancer Res.* 54:1367–1373 (1994); Arteaga et al. *Cancer Res.* 54:3758–3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160–15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMab HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin Oncol.* 14:737–744 [1996]). The recommended initial loading dose for HERCEPTIN® is 4 mg/kg administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary

disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], *supra*; Ravdin and Chamness, *Gene* 159:19–27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165–184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11 (3 Suppl 1):43–48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug

administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 $\mu\text{g/ml}$ and does not fall below 0.01 $\mu\text{g/ml}$ during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2–3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial

carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two

weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracycline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5-8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody may be an intact antibody (e.g., an intact IgG, antibody) or an antibody fragment (e.g., a Fab, F(ab')₂, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. 5A and 5B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67 (10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 µCi each of ³⁵S methionine and ³⁵S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. 2 depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO: 1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. 3 is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (µg/ml, mean ±SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. 4A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. 4B is a semi-logarithmic plot of the same data as in FIG. 4A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. 5A and 5B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. 5A) and variable heavy (V_H) (FIG. 5B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κL, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows-IleL2Thr; ArgL54Leu; TyrL55Glu; ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE
PREFERRED EMBODIMENTS

I. Definitions

An “ErbB receptor” is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms “ErbB1”, “epidermal growth factor receptor” and “EGFR” are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881–914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207–4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL RB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

“ErbB3” and “HER3” refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193–9197 (1989), including variants thereof. Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof.

The terms “ErbB4” and “HER4” herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746–1750 (1993); and Plowman et al., *Nature*, 366:473–475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms “HER2”, “ErbB2” “c-Erb-B2” are used interchangeably. Unless indicated otherwise, the terms “ErbB2” “c-Erb-B2” and “HER2” when used herein refer to the human protein, and “erbB2”, “c-erb-B2,” and “her2” refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS (USA)* 82:6497–650 (1985) and Yamamoto et al. *Nature* 319:230–234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1–4).

The “epitope 4D5” is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. 1) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The “epitope 3H4” is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This

epitope is shown in FIG. 1, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The “epitope 7C2/7F3” is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term “induces cell death” or “capable of inducing cell death” refers to the ability of the antibody to make a viable cell become nonviable. The “cell” here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which “overexpresses” ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cyto-technology* 17:1–11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the “PI uptake assay in BT474 cells”.

The phrase “induces apoptosis” or “capable of inducing apoptosis” refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the “cell” is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an “annexin binding assay using BT474 cells” (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the

US 6,627,196 B1

11

ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0–10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545–548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmuno-precipitation (RIA).

“Heregulin” (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205–1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3): 1909–1919 (1994); *Nature*, 362:312–318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. HRG β ₁₇₇₋₂₄₄).

The “ErbB2-ErbB3 protein complex” and “ErbB2-ErbB4 protein complex” are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661–14665 (1994).

“Antibodies” (Abs) and “immunoglobulins” (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

“Native antibodies” and “native immunoglobulins” are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term “variable” refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen.

12

However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91–3242, Vol. I, pages 647–669 [1991]). The constant domains involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab)_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

“Fv” is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab)_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The “light chains” of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term “antibody” is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal

US 6,627,196 B1

13

antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10): 1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 [1984]).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize

14

antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

US 6,627,196 B1

15

A “disorder” is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term “therapeutically effective amount” is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term “cytotoxic agent” as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131} , I^{125} , Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepe and cyclophosphamide (CYTOXANTM); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, carminomycin, carzinophillin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin,

16

rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitostanol, mepitostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglutone; aldophosphamide glycoside; aminolevulinic acid; amsa-crine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguanzone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK[®]; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (“Ara-C”); cyclophosphamide; thiotepe; taxanes, e.g. paclitaxel (TAXOL[®], Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE[®], Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; espermamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A “growth inhibitory agent” when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 over-expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL[®], and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled “Cell cycle regulation, oncogenes, and antineoplastic drugs” by Murakami et al. (W B Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

“Doxorubicin” is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,

US 6,627,196 B1

17

3,6-trideoxy- α -L-lyxo-hexopyranosyl]oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and β ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- β ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF- α or TNF- β ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

18

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient,

US 6,627,196 B1

19

preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/00051, published Jan. 5, 1989; PCT/US90/02697, published May 18, 1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the

20

extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS (USA)* 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

10 Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfo succinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl₂, or R¹N=C=NR, where R and R¹ are different alkyl groups.

20 Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. 25 One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are 35 suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for 40 possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or 60 more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

US 6,627,196 B1

21

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51–63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59–103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-M EM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256–262 (1993) and Plückthun, *Immunol. Revs.*, 130:151–188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779–783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al.,

22

Nuc. Acids. Res., 21:2265–2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as “import” residues, which are typically taken from an “import” variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522–525 (1986); Riechmann et al., *Nature*, 332:323–327 (1988); Verhoeven et al., *Science* 239:1534–1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such “humanized” antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called “best-fit” method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the

functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581–597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107–117(1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10: 163–167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon- α , vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537–539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory “cavities” of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or “heteroconjugate” antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target

US 6,627,196 B1

25

immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')₂ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (scFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies With the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or

26

7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM) :Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10⁶ per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca²⁺ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca²⁺ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FITC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin/streptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish

US 6,627,196 B1

27

(2 mls/35 mm dish) 2.5 μ g/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50–100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191–1195 (1992) and Shopes, B. *J. Immunol.* 148:2918–2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560–2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219–230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y and ¹⁸⁶Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridylthiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyl-diethylene triaminopentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionuclide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a “receptor” (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound

28

conjugate from the circulation using a clearing agent and then administration of a “ligand” (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionuclide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). S Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286–288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19):1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as “abzymes”, can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active

portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604–608 [1984]).

(xi) Antibody-salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNS-SMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNIS-DGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or F(ab')₂. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by

centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163–167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ 1, γ 2, or γ 4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1–13 [1983]). Protein G is recommended for all mouse isotypes and for human γ 3 (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5–4.5, preferably performed at low salt concentrations (e.g. from about 0–0.25M salt).

III. Determination of anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMab HER2 (humanized anti-p185^{HER2} monoclonal antibody, including HERCEPTIN® anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

Plate Binding Activity Assay for rhuMab HER2 (Humanized Anti-p185^{HER2} Monoclonal Antibody)

The method of assaying rhuMab HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMab HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilutions of standardized rhuMab HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

US 6,627,196 B1

31

An aliquot of Coat Antigen in Coating buffer (recombinant p185^{HER2} (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2–8° C. for 12–72 hours. The coating solution was removed and each well was washed six times with water, then blotted to remove excess water.

An aliquot of Assay Diluent was added to each well and incubated for 1–2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for 1 hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H₂O₂ in PBS) was added to each well and incubated for a sufficient period of time (approximately 8–10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490–492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURON-ICSTM or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activi-

32

ties that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's *Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment With the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as

US 6,627,196 B1

33

intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an anticycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992)*. The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or-radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 $\mu\text{g/kg}$ to 15 mg/kg (e.g. 0.1–20 mg/kg) of antibody is an initial candidate dosage for administration to the patient,

34

whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 $\mu\text{g/kg}$ to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg , 8 mg/kg , or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2–3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instruc-

tions for use, including, e.g., a warning that the composition is not to be used in combination with anthracycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.
Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody

Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 IgG₁k murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50: 1550–1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2–3₄₀₀ cells (expressing approximately 1×10⁵ ErbB2 molecules/cell) produced as described in Hudziak et al., *Proc. Natl. Acad. Sci. (USA)* 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10⁷ cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ³²P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG₁ (IgG₁) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285–4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185^{HER2} (Dissociation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of p185^{HER2}, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria

Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to over-express ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p185^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women ≥18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria

Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer
Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered

intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m²) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m²) or epirubicin (75 mg/m²) were given either by slow iv push over a minimum period of 3–5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paclitaxel (TAXOL®) was given at a dose of 175 mg/m² over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H₂ blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease

Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response

Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response

A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response

A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease

No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed.2), New York, N.Y., Wiley, 1981, pp 13–17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response

rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN® Anti-ErbB2 Antibody Efficacy				
	Enrolled	TTP (months)	RR (%)	AE (%)
CRx	234	5.5	36.2	66
CRx + 14	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test; ** p < 0.01 by X² test; CRx: chemotherapy; AC: anthracycline/cyclophosphamide treatment; H: HERCEPTIN® anti-ErbB2 antibody; T: TAXOL®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade ¾) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2

Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous, infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infusions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0–36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations (µg/ml, mean±SE) from Week 2 through Week 36 are plotted in FIG. 3 (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1–8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion

were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment (µg/ml)						
	Dose Number	n	Mean	SD	Minimum	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough		195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough		167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough		179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough		132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough		141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough		115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough		137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 µg/ml from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 µg/ml was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 (p<0.001). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60±20 µg/ml in the responders versus 44±25 µg/ml in the nonresponders (mean±SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations (n=40, mean=28.8 µg/ml, SD=10.4) compared with patients with 3+ HER2 overexpression (n=155, mean=24.1 µg/ml, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough

serum concentrations (n=12, mean 34.1 µg/ml, SD=12.0) compared with patients with visceral disease (n=183, mean=24.4 µg/ml, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of 4 mg/kg weekly. The results of this preliminary human study indicated that an 8mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3

I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN®, see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474MI, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu 7-9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10⁶ BT474M 1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm³, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion				
Group, Dose, Antibody	Target Serum Conc. µg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1-Control, rhuMab E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2-Low Dose SC rhuMab HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3-High Dose SC rhuMab HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4-IV Multi-Dose rhuMab HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum.
LD = loading dose.
MD = maintenance dose.
Infusate concentration was calculated to achieve targeted serum concentration using Alzet® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of

dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M 1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 antibody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 µg/ml or 20 µg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (Oust prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm³. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M 1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN® -treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery			
Treatment Group	Tumor Volume (mm ³), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6–Day 35 (n = 13)	HERCEPTIN® Serum Conc. (µg/ml), Day 27, (n = 3)
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery; i.v. = intravenous delivery.
*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.
**at predose (trough serum concentration immediately prior to a maintenance dose)

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 µg/ml was as effective as a concentration of 20 µg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4

I.V. Bolus and Subcutaneous Bolus Deliveries of
HERCEPTIN® Anti-ErbB2 Antibody Effectively
Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell inoculation as described in Example 3. Six days after tumor cell inoculation, the initial tumor measurement was performed. Seven days after tumor cell inoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Bolus Delivery				
Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1-Control rhuMab E25	IV	8	4	10
2-rhuMab HER2	IV	2	1	10
3-rhuMab HER2	IV	4	2	10
4-rhuMab HER2	IV	8	4	10
5-rhuMab HER2	SC	4	2	10

IV = intravenous; SC = subcutaneous; n = number of animals per group.

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery: Serum HERCEPTIN® Anti-ErbB2 Antibody Concentration Serum Concentration, µg/ml				
Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1-Control rhu MAb E25 (IV, 4mg/kg)	0 (0)	25.9 (8.29)	34.6 (11.2)	38.5 (14.4)
2-rhu MAb HER2 (IV, 1 mg/kg)	0 (0)	4.96 (3.79)	8.55 (5.83)	8.05 (4.67)
3-rhu MAb HER2 (IV, 2 mg/kg)	0 (0)	13.4 (9.24)	18.9 (12.0)	22.6 (9.21)
4-rhu MAb HER2 (IV, 4 mg/kg)	0 (0)	29.6 (13.5)	37.7 (14.4)	46.2 (13.8)
5-rhu MAb HER2 (SC, 2 mg/kg)	0 (0)	12.5 (7.33)	16.9 (10.2)	17.6 (10.7)

n = 10 for time points Days 0, 7 and 14.
N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1–5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)					
Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm ³	Tumor Vol. Day 28, mm ³	Tumor Vol. Day 31, mm ³	Day 6–Day 31* Area Under Curve Tumor Vol., mm ³	Tumor Growth Rate on Log (TM + 1)
1-IV Control	321 (190)	1530 (1040)	1630 (1170)	13600 (7230)	0.0660 (0.0200)
2-IV Herceptin 1 mg/kg	297 (130)	175 (215)	151 (188)	4690 (1400)	–0.0505 (0.142)
3-IV Herceptin 2 mg/kg	269 (129)	75.7 (92.4)	73.6 (84.5)	3510 (1220)	–0.0608 (0.110)
4-IV Herceptin 4 mg/kg	272 (117)	25.3 (75.9)	25.8 (72.9)	2880 (1230)	–0.0810 (0.0859)
5-SC Herceptin 2 mg/kg	268 (117)	76.2 (98.8)	90.4 (105)	3230 (1440)	–0.0304 (0.104)

N = 10 for each data point.
TM = tumor measurement.
IV = intravenous.
SC = subcutaneous.
MD = maintenance dose.
Tumor Vol. = tumor volume, mm³.
*Day 17 excluded due to measurement error.
Tumor growth rate calculated on Day 21–Day 31 Log(TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

FIGS. 4A and 4B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. 4A is a linear plot of tumor volume versus time. FIG. 4B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. 4A and 4B indicate that, although a dose-related response was not observed between HERCEPTIN-treated groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5

Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the invention, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1

week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10–20 µg/ml is achieved (averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10–20 µg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10–20 µg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion.

This is followed by administration of 8 mg/kg per week or 8 mg/kg per 2–3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10–20 µg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10–20 µg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® 0 anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10–20 µg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthracycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175mg/m² every 3 weeks) or an anthracycline derivative (e.g. doxorubicin 60 mg/m² or epirubicin 75 mg/m² every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m² cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70–100 mg/m²/

week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30–70 mg/m²/week.

Example 6

HERCEPTIN® Administered Intravenously Every Three Weeks in Combination with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with paclitaxel (175 mg/m² every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10–20mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

- 1) Females ≥ 18 years of age
- 2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
- 3) Patients who have been newly diagnosed with metastatic disease
- 4) Have a Karnofsky performance status of ≥ 70%
- 5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

- 1) Pregnant or lactating women
- 2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
- 3) Clinical or radiologic evidence of CNS metastases.
- 4) History of any significant cardiac disease
- 5) LVEF ≤ 50%
- 6) No prior taxane therapy in any treatment setting.
- 7) Any of the following abnormal baseline hematologic values:
 - Hb less than 9 g/dl
 - WBC less than 3.0×10⁹/l
 - Granulocytes less than 1.5×10⁹/l
 - Platelets less than 100×10⁹/l
- 8) Any of the following abnormal baseline liver function tests:
 - Serum bilirubin greater than 1.5× ULN (upper normal limit)
 - ALT and/or AST greater than 2.5× ULN (greater than 4.0× ULN if liver or bone metastasis)
 - Alkaline phosphatase greater than 2.5× ULN (greater than 4.0× ULN if liver or bone metastasis)
- 9) The following abnormal baseline renal function tests:
 - serum creatinine greater than 1.5× ULN
- 10) History of other serious medical conditions that would preclude patient participation in an investigational study.

HERCEPTIN® Loading dose and schedule: 8 mg/kg for first dose. Maintenance dose and schedule: 6 mg/kg every 3 weeks.

Paclitaxel—175 mg/m² IV every 3 weeks×6 cycles as a 3-hour infusion. NOTE: On the first cycle of treatment,

paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.

The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN® +/- paclitaxel at the discretion of the investigator.

It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.

5 While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred 10 embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

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US 6,627,196 B1

49

50

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US 6,627,196 B1

51

52

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US 6,627,196 B1

53

54

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95 100 105

Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
110 115

What is claimed is:

1. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB2 antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose, wherein

57

the subsequent doses are separated in time from each other by at least two weeks.

2. The method of claim 1, wherein the initial dose is at least approximately 6 mg/kg.

3. The method of claim 2, wherein the initial dose is at least approximately 8 mg/kg.

4. The method of claim 3, wherein the initial dose is at least approximately 12 mg/kg.

5. The method of claim 1, wherein the subsequent doses are separated in time from each other by at least three weeks.

6. The method of claim 1, wherein the initial dose is administered by intravenous injection, and wherein at least one subsequent dose is administered by subcutaneous injection.

7. The method of claim 1, wherein the initial dose is administered by intravenous injection, wherein at least two subsequent doses are administered, and wherein each subsequent dose is administered by a method selected from the group consisting of intravenous injection and subcutaneous injection.

8. The method of claim 1, wherein the initial dose and at least one subsequent dose are administered by subcutaneous injection.

9. The method of claim 1, wherein the initial dose is selected from the group consisting of approximately 6 mg/kg, 8 mg/kg, or 12 mg/kg, wherein the plurality of subsequent doses are at least approximately 2 mg/kg.

10. The method of claim 9, wherein the plurality of subsequent doses are separated in time from each other by at least three weeks.

11. The method of claim 10, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

12. The method of claim 10, wherein the initial dose is approximately 12 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

13. The method of claim 9, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 8 mg/kg.

14. The method of claim 9, wherein the initial dose is approximately 8 mg/kg, wherein at least one subsequent dose is 8 mg/kg, and wherein administration of the initial dose and subsequent doses are separated in time by at least 2 weeks.

15. The method of claim 14, wherein the initial dose and subsequent doses are separated in time by at least 3 weeks.

16. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of the antibody, wherein the initial dose is a plurality of doses, wherein each of the plurality of initial doses is at least approximately 1 mg/kg and is administered on at least 3 consecutive days, and administering to the patient at least 1 subsequent dose of the antibody, wherein at least one subsequent dose is at least approximately 6 mg/kg, and wherein administration of the last initial dose and

58

the first subsequent and additional subsequent doses are separated in time by at least 3 weeks.

17. The method of claim 1, wherein said cancer is selected from the group consisting of breast cancer, leukemia, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

18. The method of claim 17, wherein said cancer is breast cancer.

19. The method of claim 18, wherein said cancer is metastatic breast carcinoma.

20. The method of claim 1, wherein said antibody binds to the extracellular domain of the ErbB2 receptor.

21. The method of claim 20, wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

22. The method of claim 21, wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

23. The method of claim 1, wherein efficacy is measured by determining the time to disease progression or the response rate.

24. A method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by two or more subsequent doses of the antibody, wherein the subsequent doses are separated in time from each other by at least two weeks.

25. The method of claim 24, wherein the first dose and a first subsequent dose are separated from each other in time by at least about three weeks.

26. The method of claim 24, wherein the first dose and subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

27. The method of claim 26, wherein the first dose and subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

28. The method of claim 27, wherein the first dose and subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

29. The method of claim 24, wherein from about two to about ten subsequent doses of the antibody are administered to the patient.

30. The method of claim 24, wherein the subsequent doses are separated in time from each other by at least about three weeks.

31. The method of claim 24, wherein the two or more subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

32. The method of claim 24, wherein the two or more subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

33. The method of claim 24, wherein the two or more subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

* * * * *

EXHIBIT D



US007371379B2

(12) **United States Patent**
Baughman et al.

(10) **Patent No.:** **US 7,371,379 B2**
(45) **Date of Patent:** **May 13, 2008**

(54) **DOSAGES FOR TREATMENT WITH
ANTI-ERBB2 ANTIBODIES**

(75) Inventors: **Sharon A. Baughman**, Ventura, CA
(US); **Steven Shak**, Burlingame, CA
(US)

(73) Assignee: **Genentech, Inc.**, South San Francisco,
CA (US)

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Primary Examiner—Alana M. Harris
Assistant Examiner—Anne L. Holleran
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(57) **ABSTRACT**

The present invention concerns the treatment of disorders
characterized by the overexpression of ErbB2. More spe-
cifically, the invention concerns the treatment of human
patients susceptible to or diagnosed with cancer overex-
pressing ErbB2 with anti-ErbB2 antibody.

40 Claims, 5 Drawing Sheets

US 7,371,379 B2

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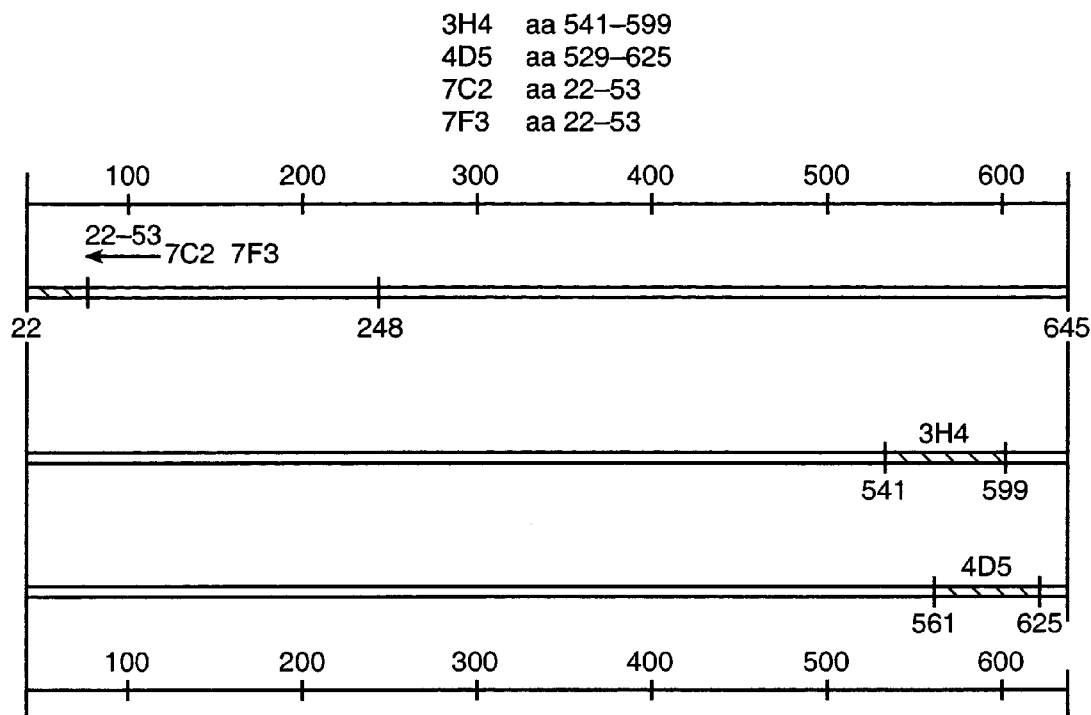
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U.S. Patent

May 13, 2008

Sheet 1 of 5

US 7,371,379 B2



3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
| 541 599

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCP SGVKPDL SYMPIWKFPDEEGACQP
| 561 625

FIG._1

1 MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPA
38 SPETHLDMLRHLYQGCQVVOGNLELTYLPTNASLSFL
75 QDIQEVQGYVLI AHNQVRQVPLQRLRIVRGTLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQRLSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTID
186 TNRSRA

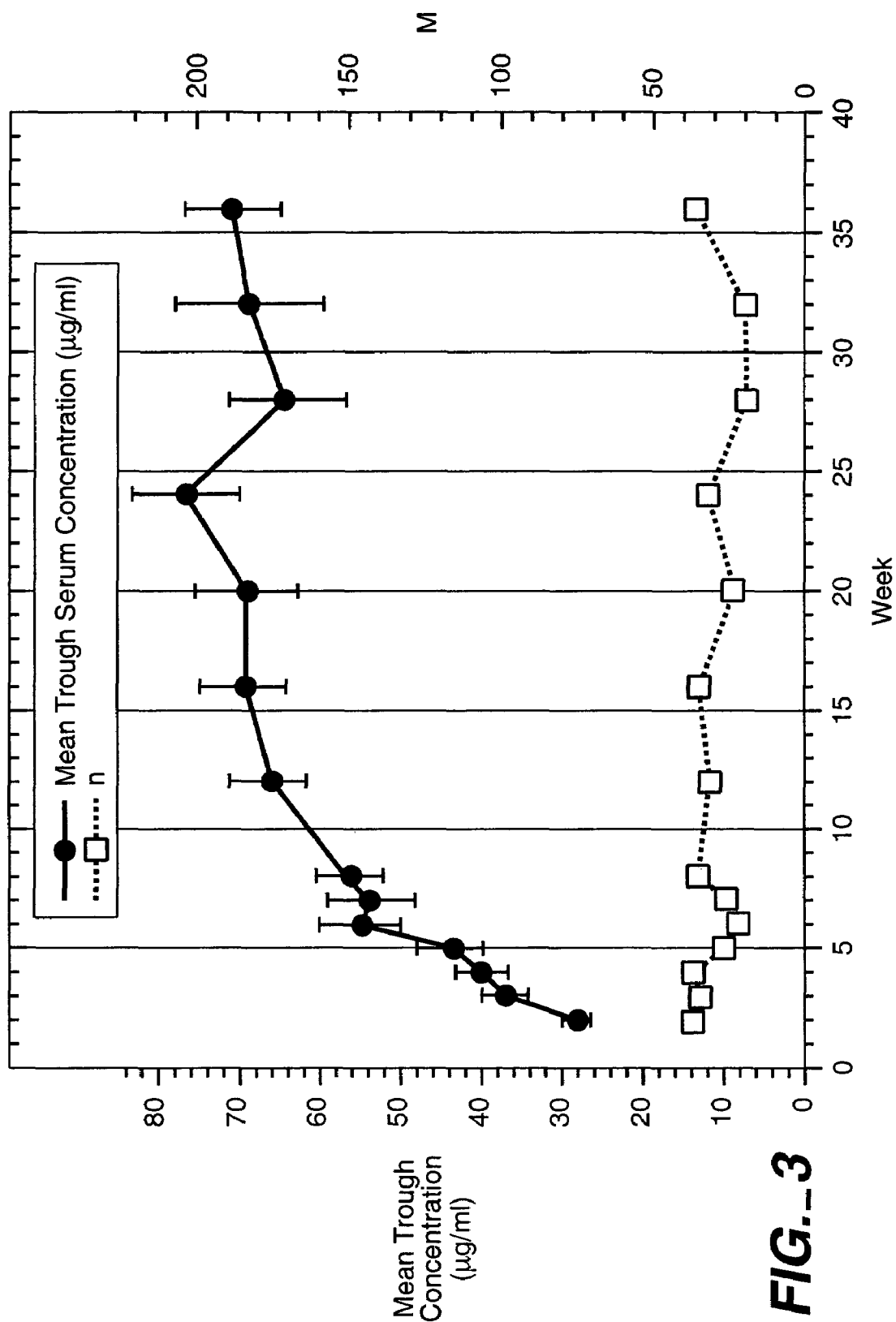
FIG._2

U.S. Patent

May 13, 2008

Sheet 2 of 5

US 7,371,379 B2



U.S. Patent

May 13, 2008

Sheet 3 of 5

US 7,371,379 B2

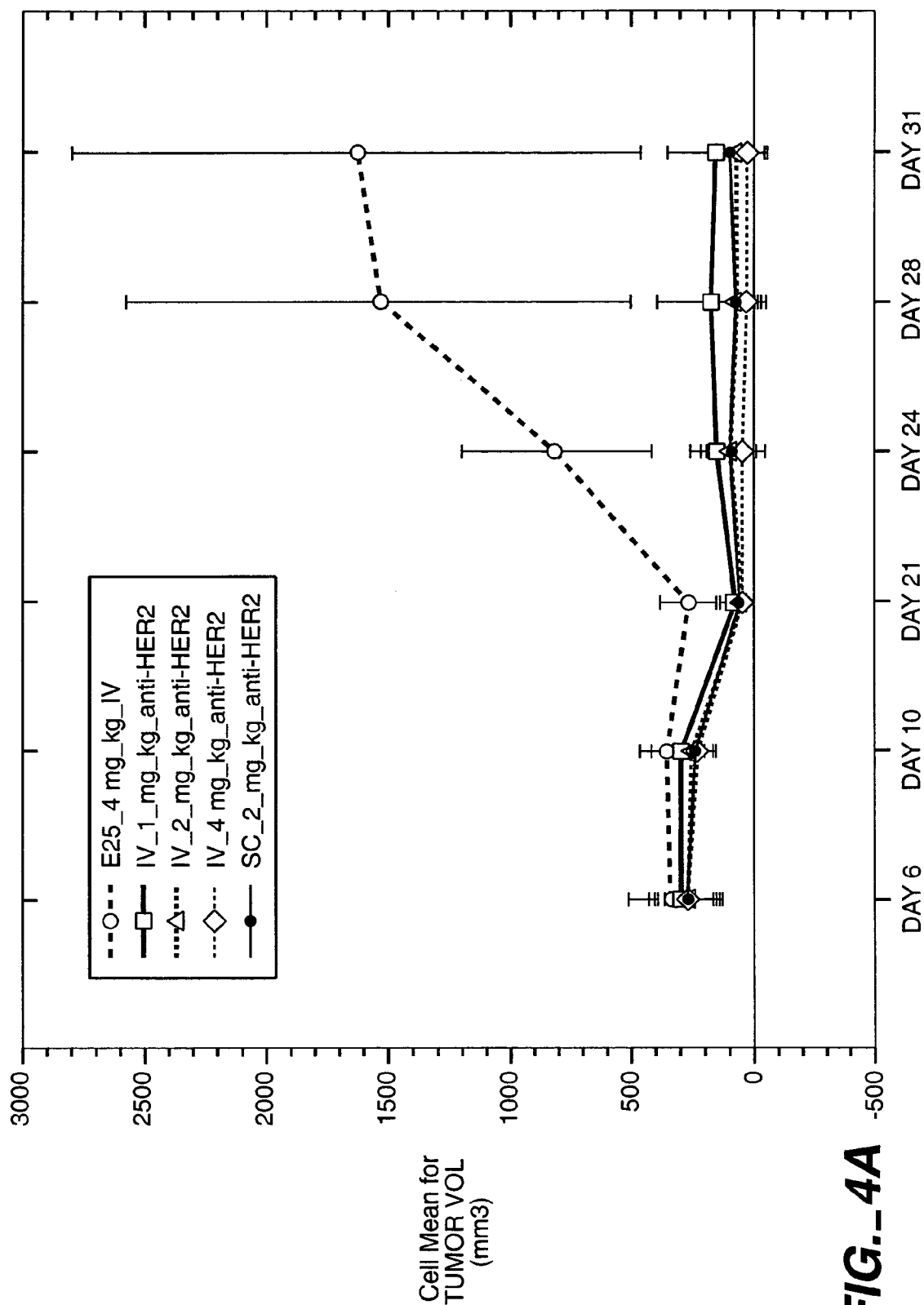


FIG. 4A

U.S. Patent

May 13, 2008

Sheet 4 of 5

US 7,371,379 B2

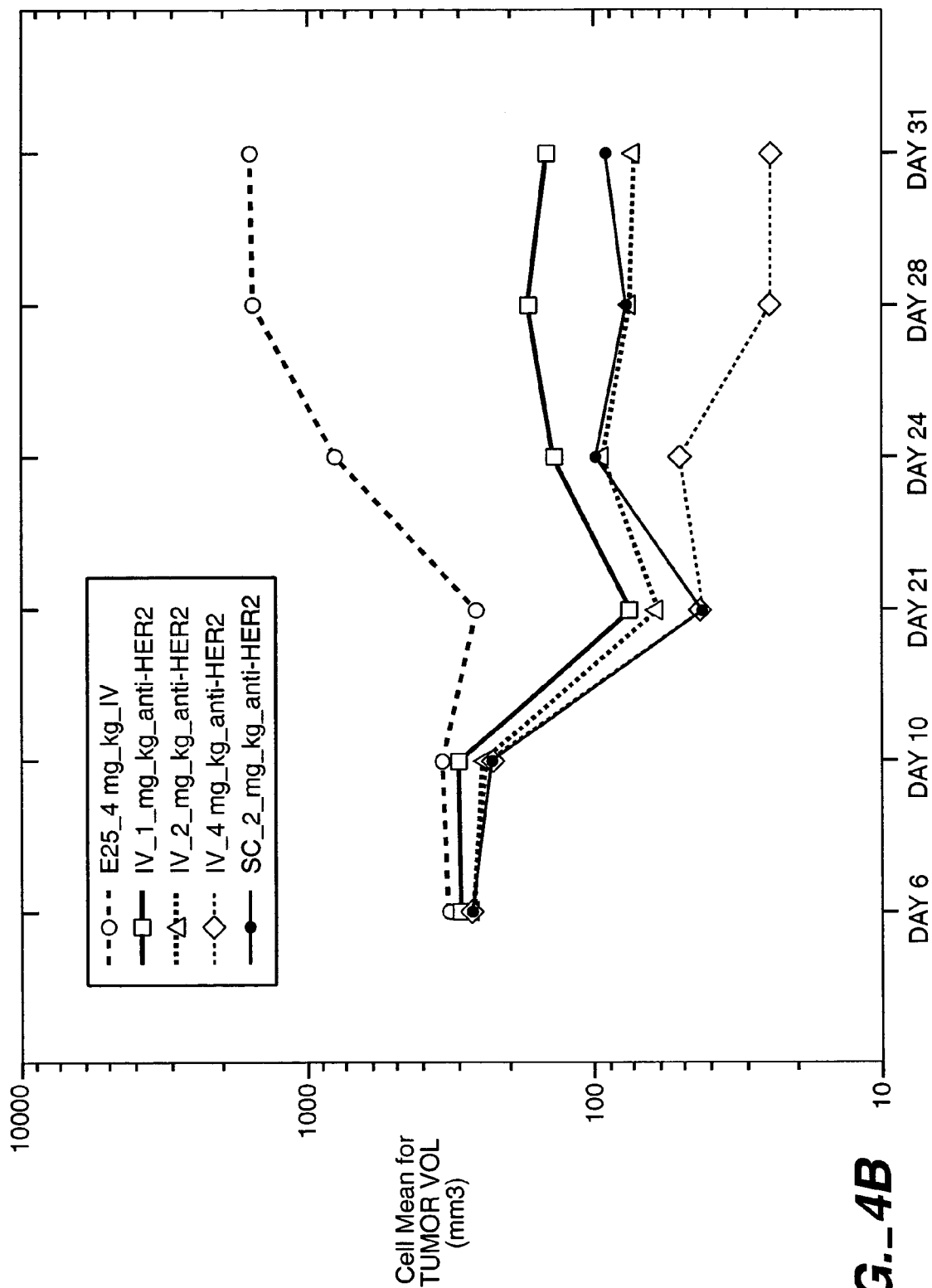


FIG. 4B

U.S. Patent

May 13, 2008

Sheet 5 of 5

US 7,371,379 B2

VARIABLE LIGHT

	1	10	20	30	40
2C4	DTVMTQSHKIMSTSVGDRVSITC [KASQDV SIGVA-----] WYQQRP				
	**	**** *	*		*
574	DIQMTQSPSSLSASVGDRVITIC [KASQDV SIGVA-----] WYQQKP				
			* * ****		
hum kI	DIQMTQSPSSLSASVGDRVITIC [RASQSVSTSSYSYMH] WYQQKP				
		50	60	70	80
2C4	GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA				
	**		* *	*	* *
574	GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISLQP				
		* ****			
hum kI	GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISLQP				
		90	100		
2C4	EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:10)				
	* *		* *		
574	EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:12)				

hum kI	EDFATYYC [QQYNSLPYT] FGQGTKVEIK (SEQ ID NO:14)				

FIG._5A

VARIABLE HEAVY

	1	10	20	30	40
2C4	EVQLQQSGPELVKPGTSVKISCKAS [GFTFTD YTMD] WVKQS				
	**	** *	**** *		*
574	EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTD YTMD] WVRQA				
			** * *		
humIII	EVQLVESGGGSVQPGGSLRLSCAAS [GFTFSS YAMS] WVRQA				
		50	60	70	80
2C4	HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM				
	* *	**		*** *	**** *
574	PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL				
	*	*****	** *****	*. * *	
humIII	PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDDSKNTLYL				
		90	100	110	
2C4	ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:11)				
	***	**		*	
574	QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:13)				
		** ***			
humIII	QMNSLRAEDTAVYYCAR [GRGGGS--DY] WGQGTTLVTSS (SEQ ID NO:15)				

FIG._5B

US 7,371,379 B2

1

**DOSAGES FOR TREATMENT WITH
ANTI-ERBB2 ANTIBODIES****RELATED APPLICATIONS**

This application is divisional of U.S. Ser. No. 09/648,067 filed Aug. 25, 2000 (now U.S. Pat. No. 6,627,196), which claims priority under 35 USC 119(e) to provisional application Nos. 60/151,018, filed Aug. 27, 1999 and 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor (p185^{HER2}) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:78-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the neu proto-oncogene) a inhibits colony formation of

2

these cells. In Drebin et al. *PNAS (USA)* 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994. Hudziak et al., *Mol. Cell. Biol.* 9(3): 1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p185^{erbB2}-overexpressing breast tumor cell lines to the cytotoxic effects of TNF- α . See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells

US 7,371,379 B2

3

was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS (USA)* 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMab HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]). The recommended initial loading dose for HERCEPTIN® is 4 mg/kg administered as a 90-minute infusion. The recom-

4

mended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluorouracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Supp11):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the

US 7,371,379 B2

5

trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 µg/ml and does not fall below 0.01 µg/ml during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gas-

6

trointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an

US 7,371,379 B2

7

anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracycline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5-8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The antibody may be an intact antibody (e.g., an intact IgG₁ anti-

8

body) or an antibody fragment (e.g., a Fab, F(ab')₂, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. 5A and 5B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative Mabs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 µCi each of ³⁵S methionine and ³⁵S cysteine. Supernatants were harvested either the ErbB2 MAb or control antibodies were added to the supernatant and incubated 24 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electrophoretically transferred onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. 2 depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAb 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. 3 is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (µg/ml, mean±SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. 4A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. 4B is a semi-logarithmic plot of the same data as in FIG. 4A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. 5A and 5B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. 5A) and variable heavy (V_H) (FIG. 5B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g. substituted as follows—IleL2Thr; ArgL54Leu; TyrL55Glu;

ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An “ErbB receptor” is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms “ErbB1”, “epidermal growth factor receptor” and “EGFR” are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

“ErbB3” and “HER3” refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193-9197 (1989), including variants thereof. Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof.

The terms “ErbB4” and “HER4” herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and Plowman et al., *Nature*, 366:473-475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms “HER2”, “ErbB2” “c-Erb-B2” are used interchangeably. Unless indicated otherwise, the terms “ErbB2” “c-Erb-B2” and “HER2” when used herein refer to the human protein, and “erbB2,” “c-erb-B2,” and “her2” refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The “epitope 4D5” is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can

be performed. Alternatively, epitope mapping can be performed (see FIG. 1) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The “epitope 3H4” is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. 1, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The “epitope 7C2/7F3” is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO: 2).

The term “induces cell death” or “capable of inducing cell death” refers to the ability of the antibody to make a viable cell become nonviable. The “cell” here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which “overexpresses” ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cyto-technology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the “PI uptake assay in BT474 cells”.

The phrase “induces apoptosis” or “capable of inducing apoptosis” refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the “cell” is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5

US 7,371,379 B2

11

to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the erbB1, erbB3 and/or erbB4 genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3):1909-1919 (1994); and Marchionni et al., *Nature*, 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. HRG β ₁₇₇₋₂₄₄).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid

12

residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. 1, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these maybe further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ ,

US 7,371,379 B2

13

respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term “antibody” is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

“Antibody fragments” comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The “monoclonal antibodies” may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include “chimeric” antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 [1984]).

“Humanized” forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the

14

human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

“Single-chain Fv” or “sFv” antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term “diabodies” refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term “salvage receptor binding epitope” refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

“Treatment” refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of

US 7,371,379 B2

15

treatment include those already with the disorder as well as those in which the disorder is to be prevented.

“Mammal” for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A “disorder” is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term “therapeutically effective amount” is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term “cytotoxic agent” as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131} , I^{125} , Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof

A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiopeta and cyclophosphamide (CYTOXANTM); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabacin,

16

carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcello-mycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitostanol, mepitostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidaniine; mitoguanzone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK[®]; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (“Ara-C”); cyclophosphamide; thiopeta; taxanes, e.g. paclitaxel (TAXOL[®], Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE[®], Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A “growth inhibitory agent” when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 over-expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL[®], and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled “Cell cycle regulation, oncogenes, and antineoplastic drugs” by Murakami et al. (W B Saunders:

US 7,371,379 B2

17

Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; prolactin; relaxin; prolactin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and - β ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- β ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF- α or TNF- β ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid

18

phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery

from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient, preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/0005 1, published Jan. 5, 1989; PCT/US90/02697, published May 18, 1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9, 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The ErbB2 antigen to be used for pro-

duction of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS (USA)* 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl₂, or R¹N=C=NR, where R and R¹ are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypox-

US 7,371,379 B2

21

anthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opin. in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 [1992]), as well as combinatorial

22

infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al. *Science*, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable

three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al. *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10: 163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope

hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H2 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or “heteroconjugate” antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab' -TNB derivatives is then reconverted to the Fab' -thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab' -TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab' -SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The “diabody” technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the “PI uptake assay using BT474 cells”. According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco’s Modified Eagle Medium (D-MEM): Ham’s F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μ g/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μ g/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an “annexin binding assay using BT474 cells” is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μ g/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μ g/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a “DNA staining assay using BT474 cells” is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μ g/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

US 7,371,379 B2

27

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin/streptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 µg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, croton, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y and ¹⁸⁶Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methylthiethylamine

28

taacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionucleotide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19):1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratin protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature*

328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g. by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNS-SMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNIS-DGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or F(ab')₂. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)

(5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSHLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABXTM resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSETM chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Determination of anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMAb HER2 (humanized anti-p185^{HER2} monoclonal antibody, including HERCEPTIN[®] anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

US 7,371,379 B2

31

Plate Binding Activity Assay for rhuMAb HER2 (Humanized Anti-p185^{HER2} Monoclonal Antibody)

The method of assaying rhuMAb HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMAb HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilutions of standardized rhuMAb HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (recombinant p185^{HER2} (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2-8° C. for 12-72 hours. The coating solution was removed and each well was washed six times with water, then blotted to remove excess water.

An aliquot of Assay Diluent was added to each well and incubated for 1-2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for 1 hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H₂O₂ in PBS) was added to each well and incubated for a sufficient period of time (approximately 8-10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490-492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16 th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbo-

32

hydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURON-ICTM or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16 th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disor-

US 7,371,379 B2

33

ders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an anthracycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered

34

for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container

US 7,371,379 B2

35

holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instructions for use, including, e.g., a warning that the composition is not to be used in combination with anthacycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRT 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody Materials and Methods

Anti-ErbB2 monoclonal antibody The anti-ErbB2 IgG₁κ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3₄₀₀ cells (expressing approximately 1×10⁵ ErbB2 molecules/cell) produced as described in Hudziak et al., *Proc. Natl. Acad. Sci. (USA)* 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10⁷ cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ³²P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus

36

human immunoglobulin IgG₁ (IgG₁) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185^{HER2} (Dissociation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of p185^{HER2}, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to over-express ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p185^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women>18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Kamofsky scale

US 7,371,379 B2

37

Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m²) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m²) or epirubicin (75 mg/m²) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paclitaxel (TAXOL®) was given at a dose of 175 mg/m² over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H₂ blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

38

Partial Response A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN® Anti-ErbB2 Antibody Efficacy				
	Enrolled	TTP(months)	RR(%)	AE(%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test;

**p < 0.01 by X² test;

CRx: chemotherapy;

AC: anthracycline/cyclophosphamide treatment;

H: HERCEPTIN® anti-ErbB2 antibody;

T: TAXOL®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2

Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infu-

sions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® Anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0-36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations ($\mu\text{g/ml}$, mean \pm SE) from Week 2 through Week 36 are plotted in FIG. 3 (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1-8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment ($\mu\text{g/ml}$)						
	Dose Number	n	Mean	SD	Minimum	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough	1	195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough	2	167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough	3	179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough	4	132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough	5	141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough	6	115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough	7	137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 $\mu\text{g/ml}$ from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 $\mu\text{g/ml}$ was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 ($p<0.001$). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60 ± 20 $\mu\text{g/ml}$ in the responders versus 44 ± 25 $\mu\text{g/ml}$ in the nonresponders (mean \pm SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations ($n=40$, mean=28.8 $\mu\text{g/ml}$, SD=10.4) compared with patients with 3+ HER2 overexpression ($n=155$, mean=24.1 $\mu\text{g/ml}$, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough serum concentrations ($n=12$, mean 34.1 $\mu\text{g/ml}$, SD=12.0) compared with patients with visceral disease ($n=183$, mean=24.4 $\mu\text{g/ml}$, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of 4 mg/kg weekly. The results of this preliminary human study indicated that an 8 mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3

I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN®, see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474M1, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu

US 7,371,379 B2

41

7-9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10^6 BT474M1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm³, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion				
Group, Dose, Antibody	Target Serum Conc. µg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1 - Control, rhuMAb E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2 - Low Dose SC rhuMAb HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3 - High Dose SC rhuMAb HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4 - IV Multi-Dose rhuMAb HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum. LD = loading dose. MD = maintenance dose. Infusate concentration was calculated to achieve targeted serum concentration using Alzet® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M 1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 antibody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 µg/ml or 20 µg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (just prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm³. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M 1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN®-treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery				
Treatment Group	Tumor Volume (mm ³), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN® Serum Conc. (µg/ml), Day 27, (n = 3)	
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94	

42

TABLE 4-continued

Comparison of S.C. Infusion and I.V. Bolus Delivery			
Treatment Group	Tumor Volume (mm ³), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN® Serum Conc. (µg/ml), Day 27, (n = 3)
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery; i.v. = intravenous delivery.

*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.

**at predose (trough serum concentration immediately prior to a maintenance dose)

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 µg/ml was as effective as a concentration of 20 µg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4

I.V. Bolus and Subcutaneous Bolus Deliveries of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell inoculation as described in Example 3. Six days after tumor cell inoculation, the initial tumor measurement was performed. Seven days after tumor cell inoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Bolus Delivery				
Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1 - Control rhuMAb E25	IV	8	4	10
2 - rhuMAb HER2	IV	2	1	10
3 - rhuMAb HER2	IV	4	2	10
4 - rhuMAb HER2	IV	8	4	10
5 - rhuMAb HER2	SC	4	2	10

IV = intravenous; SC = subcutaneous; n = number of animals per group.

US 7,371,379 B2

43

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery: Serum HERCEPTIN® Anti-ErbB2 Antibody Concentration				
Treatment Group (delivery, MD)	Serum Concentration, µg/ml			
	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1 - Control rhu MAb E25 (IV, 4 mg/kg)	0 (0)	25.9 (8.29)	34.6 (11.2)	38.5 (14.4)
2 - rhu MAb HER2 (IV, 1 mg/kg)	0 (0)	4.96 (3.79)	8.55 (5.83)	8.05 (4.67)
3 - rhu MAb HER2 (IV, 2 mg/kg)	0 (0)	13.4 (9.24)	18.9 (12.0)	22.6 (9.21)
4 - rhu MAb HER2 (IV, 4 mg/kg)	0 (0)	29.6 (13.5)	37.7 (14.4)	46.2 (13.8)
5 - rhu MAb HER2 (SC, 2 mg/kg)	0 (0)	12.5 (7.33)	16.9 (10.2)	17.6 (10.7)

n = 10 for time points Days 0, 7 and 14. N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1-5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)					
Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm ³	Tumor Vol. Day 28, mm ³	Tumor Vol. Day 31, mm ³	Day 6-Day 31* Area Under Curve Tumor Vol., mm ³	Tumor Growth Rate on Log (TM + 1)
1-IV Control	321 (190)	1530 (1040)	1630 (1170)	13600 (7230)	0.0660 (0.0200)
2-IV Herceptin 1 mg/kg	297 (130)	175 (215)	151 (188)	4690 (1400)	-0.0505 (0.142)
3-IV Herceptin 2 mg/kg	269 (129)	75.7 (92.4)	73.6 (84.5)	3510 (1220)	-0.0608 (0.110)
4-IV Herceptin 4 mg/kg	272 (117)	25.3 (75.9)	25.8 (72.9)	2880 (1230)	-0.0810 (0.0859)
5-SC Herceptin 2 mg/kg	268 (117)	76.2 (98.8)	90.4 (105)	3230 (1440)	-0.0304 (0.104)

N = 10 for each data point. TM = tumor measurement. IV = intravenous. SC = subcutaneous. MD = maintenance dose. Tumor Vol. = tumor volume, mm³.

*Day 17 excluded due to measurement error.

Tumor growth rate calculated on Day 21-Day 31 Log (TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

FIGS. 4A and 4B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. 4A is a linear plot of tumor volume versus time. FIG. 4B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. 4A and 4B indicate that, although a dose-related response was not observed between HERCEPTIN-treated

44

groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5

Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the invention, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a

60

decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

65

US 7,371,379 B2

45

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 µg/ml is achieved (averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 µg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 µg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion. This is followed by administration of 8 mg/kg per week or 8 mg/kg per 2-3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 µg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10-20 µg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions

46

per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10-20 µg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthracycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175 mg/m² every 3 weeks) or an anthracycline derivative (e.g. doxorubicin 60 mg/m² or epirubicin 75 mg/m² every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m² cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70-100 mg/m²/week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30-70 mg/m²/week.

Example 6

HERCEPTIN® Administered Intravenously Every Three Weeks in Combination with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with

US 7,371,379 B2

47

paclitaxel (175 mg/m² every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10-20 mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

- 1) Females 24-18 years of age
- 2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
- 3) Patients who have been newly diagnosed with metastatic disease
- 4) Have a Karnofsky performance status of 24-70%
- 5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

- 1) Pregnant or lactating women
- 2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
- 3) Clinical or radiologic evidence of CNS metastases.
- 4) History of any significant cardiac disease
- 5) LVEF ≤ 50%
- 6) No prior taxane therapy in any treatment setting.
- 7) Any of the following abnormal baseline hematologic values:
 - Hb less than 9 g/dl
 - WBC less than 3.0×10⁹/l
 - Granulocytes less than 1.5×10⁹/l
 - Platelets less than 100×10⁹/l
- 8) Any of the following abnormal baseline liver function tests:
 - Serum bilirubin greater than 1.5×ULN (upper normal limit)
 - ALT and/or AST greater than 2.5×ULN (greater than 4.0×ULN if liver or bone metastasis)

48

Alkaline phosphatase greater than 2.5×ULN (greater than 4.0×ULN if liver or bone metastasis)

- 9) The following abnormal baseline renal function tests:

serum creatinine greater than 1.5×ULN

- 10) History of other serious medical conditions that would preclude patient participation in an investigational study.

HERCEPTIN® Loading dose and schedule: 8 mg/kg for first dose. Maintenance dose and schedule: 6 mg/kg every 3 weeks.

Paclitaxel—175 mg/m² IV every 3 weeks×6 cycles as a 3-hour infusion.

NOTE: On the first cycle of treatment, paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.

The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN® +/- paclitaxel at the discretion of the investigator.

It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.

While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

SEQUENCE LISTING

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20 25 30

Val Gln Gly Asn Leu Glu Leu Thr Tyr Leu Pro Thr Asn Ala Ser
35 40 45

Leu Ser Phe Leu Gln Asp Ile Gln Glu Val Gln Gly Tyr Val Leu
50 55 60

US 7,371,379 B2

49

50

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Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
      65                      70                      75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
      80                      85                      90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
      95                      100                     105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
      110                     115                     120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
      125                     130                     135

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys
      140                     145                     150

Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg
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Ala

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<400> SEQUENCE: 2

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Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His Leu Tyr Gln
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Gly Cys

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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: salvage receptor binding epitope

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<400> SEQUENCE: 4

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His Gln Ser Leu Gly Thr Gln
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<220> FEATURE:
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US 7,371,379 B2

51

52

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<210> SEQ ID NO 6
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 <213> ORGANISM: Artificial sequence
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 <223> OTHER INFORMATION: salvage receptor binding epitope

<400> SEQUENCE: 6

His Gln Asn Ile Ser Asp Gly Lys
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<210> SEQ ID NO 7
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 <212> TYPE: PRT
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 <220> FEATURE:
 <223> OTHER INFORMATION: salvage receptor binding epitope

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Val Ile Ser Ser His Leu Gly Gln
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<210> SEQ ID NO 8
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Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
 1 5 10 15
 Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
 20 25 30
 Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
 35 40 45
 Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg
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<210> SEQ ID NO 9
 <211> LENGTH: 65
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 <213> ORGANISM: Homo sapiens

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 1 5 10 15
 Cys Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr
 20 25 30
 Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys
 35 40 45
 Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu
 50 55 60
 Gly Ala Cys Gln Pro
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 <212> TYPE: PRT
 <213> ORGANISM: Mus Musculus

US 7,371,379 B2

53

54

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				20					25					30

Ile	Gly	Val	Ala	Trp	Tyr	Gln	Gln	Arg	Pro	Gly	Gln	Ser	Pro	Lys
				35					40					45

Leu	Leu	Ile	Tyr	Ser	Ala	Ser	Tyr	Arg	Tyr	Thr	Gly	Val	Pro	Asp
				50					55					60

Arg	Phe	Thr	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Phe	Thr	Ile
				65					70					75

Ser	Ser	Val	Gln	Ala	Glu	Asp	Leu	Ala	Val	Tyr	Tyr	Cys	Gln	Gln
				80					85					90

Tyr	Tyr	Ile	Tyr	Pro	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu
				95					100					105

Ile Lys

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<211> LENGTH: 119

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 11

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Thr	Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Thr	Phe	Thr
				20					25					30

Asp	Tyr	Thr	Met	Asp	Trp	Val	Lys	Gln	Ser	His	Gly	Lys	Ser	Leu
				35					40					45

Glu	Trp	Ile	Gly	Asp	Val	Asn	Pro	Asn	Ser	Gly	Gly	Ser	Ile	Tyr
				50					55					60

Asn	Gln	Arg	Phe	Lys	Gly	Lys	Ala	Ser	Leu	Thr	Val	Asp	Arg	Ser
				65					70					75

Ser	Arg	Ile	Val	Tyr	Met	Glu	Leu	Arg	Ser	Leu	Thr	Phe	Glu	Asp
				80					85					90

Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Asn	Leu	Gly	Pro	Ser	Phe	Tyr
				95					100					105

Phe	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Thr	Leu	Thr	Val	Ser	Ser
				110					115				

<210> SEQ ID NO 12

<211> LENGTH: 107

<212> TYPE: PRT

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: humanized VL sequence

<400> SEQUENCE: 12

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1				5					10					15

Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Lys	Ala	Ser	Gln	Asp	Val	Ser
				20					25					30

Ile	Gly	Val	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys
				35					40					45

Leu	Leu	Ile	Tyr	Ser	Ala	Ser	Tyr	Arg	Tyr	Thr	Gly	Val	Pro	Ser
				50					55					60

US 7,371,379 B2

55

56

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Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile
				65					70					75
Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln
				80					85					90
Tyr	Tyr	Ile	Tyr	Pro	Tyr	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu
				95					100					105

Ile Lys

<210> SEQ ID NO 13
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 <212> TYPE: PRT
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: humanized VH sequence

<400> SEQUENCE: 13

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Gly	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Thr
				20					25					30
Asp	Tyr	Thr	Met	Asp	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
				35					40					45
Glu	Trp	Val	Ala	Asp	Val	Asn	Pro	Asn	Ser	Gly	Gly	Ser	Ile	Tyr
				50					55					60
Asn	Gln	Arg	Phe	Lys	Gly	Arg	Phe	Thr	Leu	Ser	Val	Asp	Arg	Ser
				65					70					75
Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp
				80					85					90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Asn	Leu	Gly	Pro	Ser	Phe	Tyr
				95					100					105
Phe	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	
				110					115					

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 <212> TYPE: PRT
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: VL consensus sequence

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Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val
1				5					10					15
Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Ser	Ile	Ser
				20					25					30
Asn	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys
				35					40					45
Leu	Leu	Ile	Tyr	Ala	Ala	Ser	Ser	Leu	Glu	Ser	Gly	Val	Pro	Ser
				50					55					60
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile
				65					70					75
Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln
				80					85					90
Tyr	Asn	Ser	Leu	Pro	Trp	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu
				95					100					105

Ile Lys

US 7,371,379 B2

57

58

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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: VH consensus sequence

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 20             25             30
Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35             40             45
Glu Trp Val Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
 50             55             60
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
 65             70             75
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80             85             90
Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
 95             100            105
Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 110            115

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The invention claimed is:

1. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising:

administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB2 antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks; and

further comprising administering an effective amount of a chemotherapeutic agent to the patient.

2. The method of claim 1, wherein the initial dose is at least approximately 6 mg/kg.

3. The method of claim 2, wherein the initial dose is at least approximately 8 mg/kg.

4. The method of claim 3, wherein the initial dose is at least approximately 12 mg/kg.

5. The method of claim 1, wherein the subsequent doses are separated in time from each other by at least three weeks.

6. The method of claim 1, wherein the initial dose is administered by intravenous injection, and wherein at least one subsequent dose is administered by subcutaneous injection.

7. The method of claim 1, wherein the initial dose is administered by intravenous injection, wherein at least two subsequent doses are administered, and wherein each subsequent dose is administered by a method selected from the group consisting of intravenous injection and subcutaneous injection.

8. The method of claim 1, wherein the initial dose and at least one subsequent dose are administered by subcutaneous injection.

9. The method of claim 1, wherein the initial dose is selected from the group consisting of approximately 6 mg/kg, 8 mg/kg, or 12 mg/kg, wherein the plurality of subsequent doses are at least approximately 2 mg/kg.

10. The method of claim 9, wherein the plurality of subsequent doses are separated in time from each other by at least three weeks.

11. The method of claim 10, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

12. The method of claim 10, wherein the initial dose is approximately 12 mg/kg, and wherein at least one subsequent dose is approximately 6 mg/kg.

13. The method of claim 9, wherein the initial dose is approximately 8 mg/kg, and wherein at least one subsequent dose is approximately 8 mg/kg.

14. The method of claim 9, wherein the initial dose is approximately 8 mg/kg, wherein at least one subsequent dose is 8 mg/kg, and wherein administration of the initial dose and subsequent doses are separated in time by at least 2 weeks.

15. The method of claim 14, wherein the initial dose and subsequent doses are separated in time by at least 3 weeks.

16. The method of claim 1, wherein said cancer is selected from the group consisting of breast cancer, leukemia, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

17. The method of claim 16, wherein said cancer is breast cancer.

US 7,371,379 B2

59

18. The method of claim 17, wherein said cancer is metastatic breast carcinoma.

19. The method of claim 1, wherein said antibody binds to the extracellular domain of the ErbB2 receptor.

20. The method of claim 19, wherein said antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

21. The method of claim 20, wherein said antibody is a humanized 4D5 anti-ErbB2 antibody.

22. The method of claim 1, wherein the chemotherapeutic agent is a taxoid.

23. The method of claim 22, wherein said taxoid is paclitaxel or docetaxel.

24. The method of claim 1, wherein the effective amount of the anti-ErbB2 antibody and the effective amount of the chemotherapeutic agent as a combination is lower than the sum of the effective amounts of said anti-ErbB2 antibody and said chemotherapeutic agent, when administered individually, as single agents.

25. The method of claim 1, wherein the chemotherapeutic agent is an anthracycline.

26. The method of claim 25, wherein the anthracycline is doxorubicin or epirubicin.

27. The method of claim 25, wherein the method further comprises administration of a cardioprotectant.

28. The method of claim 1, wherein efficacy is measured by determining the time to disease progression or the response rate.

29. A method for the treatment of a human patient diagnosed with cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of an anti-ErbB2 antibody to the human patient, the method comprising: administering to the patient an initial dose of the antibody, wherein the initial dose is a plurality of doses, wherein each of the plurality of initial doses is at least approximately 1 mg/kg and is administered on at least 3 consecutive days, and administering to the patient at least one subsequent dose of the antibody, wherein at least one subsequent dose is at least approximately 6 mg/kg, and wherein administration of the last initial dose and the first subsequent and additional subsequent doses are separated in

60

time by at least 3 weeks, and further comprising administering an effective amount of a chemotherapeutic agent to the patient.

30. A method for the treatment of cancer in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by two or more subsequent doses of the antibody, wherein the subsequent doses are separated from each other in time by at least about two weeks, and further comprising administering an effective amount of a chemotherapeutic agent to the patient.

31. The method of claim 30, wherein the first dose and a first subsequent dose are separated from each other in time by at least about three weeks.

32. The method of claim 30, wherein the first dose and subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

33. The method of claim 32, wherein the first dose and subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

34. The method of claim 33, wherein the first dose and subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

35. The method of claim 30, wherein from about two to about ten subsequent doses of the antibody are administered to the patient.

36. The method of claim 30, wherein the two or more subsequent doses are separated from each other in time by at least about three weeks.

37. The method of claim 30, wherein the two or more subsequent doses are each from about 2 mg/kg to about 16 mg/kg.

38. The method of claim 30, wherein the two or more subsequent doses are each from about 4 mg/kg to about 12 mg/kg.

39. The method of claim 30, wherein the two or more subsequent doses are each from about 6 mg/kg to about 12 mg/kg.

40. The method of claim 30, wherein the chemotherapeutic agent is a taxoid.

* * * * *

EXHIBIT E



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(12) **United States Patent**
Baughman et al.

(10) **Patent No.: US 10,160,811 B2**
(45) **Date of Patent: *Dec. 25, 2018**

(54) **TREATMENT WITH ANTI-ERBB2
ANTIBODIES**

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- (71) Applicant: **GENENTECH, INC.**, South San Francisco, CA (US)
- (72) Inventors: **Sharon A. Baughman**, Ventura, CA (US); **Steven Shak**, Burlingame, CA (US)
- (73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)
- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 843 days.

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Primary Examiner — Ilia I Ouspenski
(74) *Attorney, Agent, or Firm* — Diane L. Marschang;
Ginger R. Dreger

(57) **ABSTRACT**

The present invention concerns dosages for treatment of human cancer patients with an anti-Epidermal Growth Factor Receptor (EGFR) antibody.

12 Claims, 5 Drawing Sheets

Specification includes a Sequence Listing.

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Page 7

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US 10,160,811 B2

Page 8

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US 10,160,811 B2

Page 9

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US 10,160,811 B2

Page 10

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US 10,160,811 B2

Page 11

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US 10,160,811 B2

Page 12

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US 10,160,811 B2

Page 13

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US 10,160,811 B2

Page 14

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US 10,160,811 B2

Page 15

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US 10,160,811 B2

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Page 17

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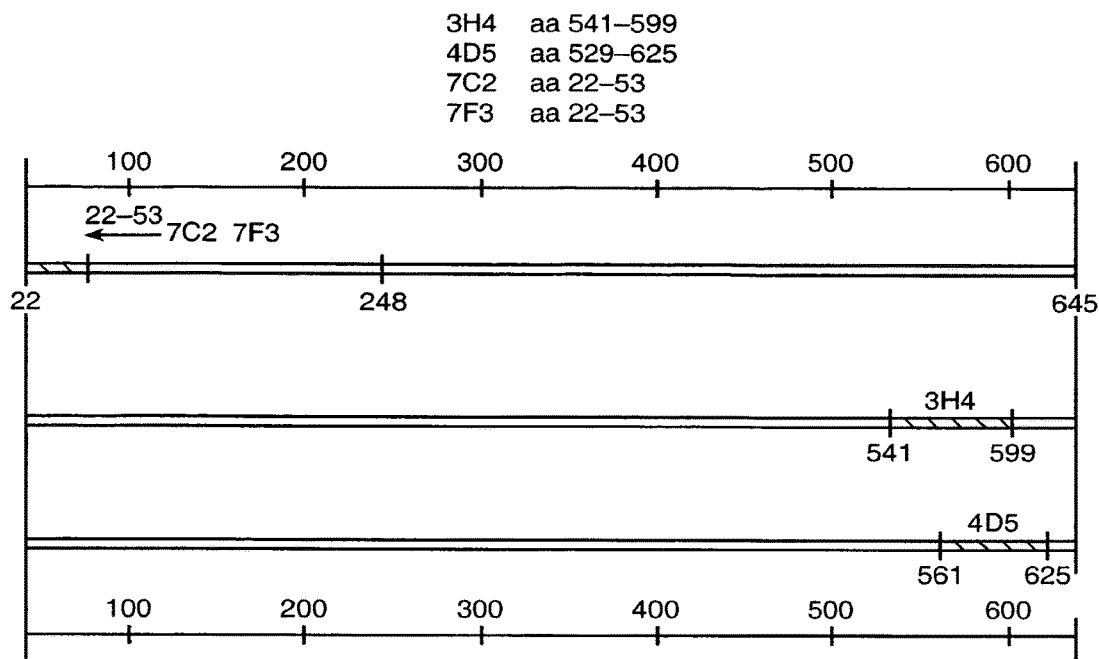
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U.S. Patent

Dec. 25, 2018

Sheet 1 of 5

US 10,160,811 B2



3H4 epitope (SEQ ID NO:8) 58 residues

VEECRVLQGLPREYVNAHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
| 541 599

4D5 epitope (SEQ ID NO:9) 64 residues

LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPGKPDLSYMPIWKFPDEEGACQP
| 561 625

FIG._1

1 MELAALCRWGLLLALLPPGA**ASTQVCTG**TDMLRLPA
38 **SPETHLDMLRHLYQGC**QVVQGNLELTYPNLSLFL
75 **QDIQEVQGYVLI**AHNQVRQVPLQRLRIVRGTLFEDN
112 **YALAVLDNGDPLN**NTTPVTGASPGGLRELQRLSLTEI
149 **LKGGVLIQRNPQ**LCYQDTILWKDIFHKNNQLALTLID
186 **TNRSRA**

FIG._2

U.S. Patent

Dec. 25, 2018

Sheet 2 of 5

US 10,160,811 B2

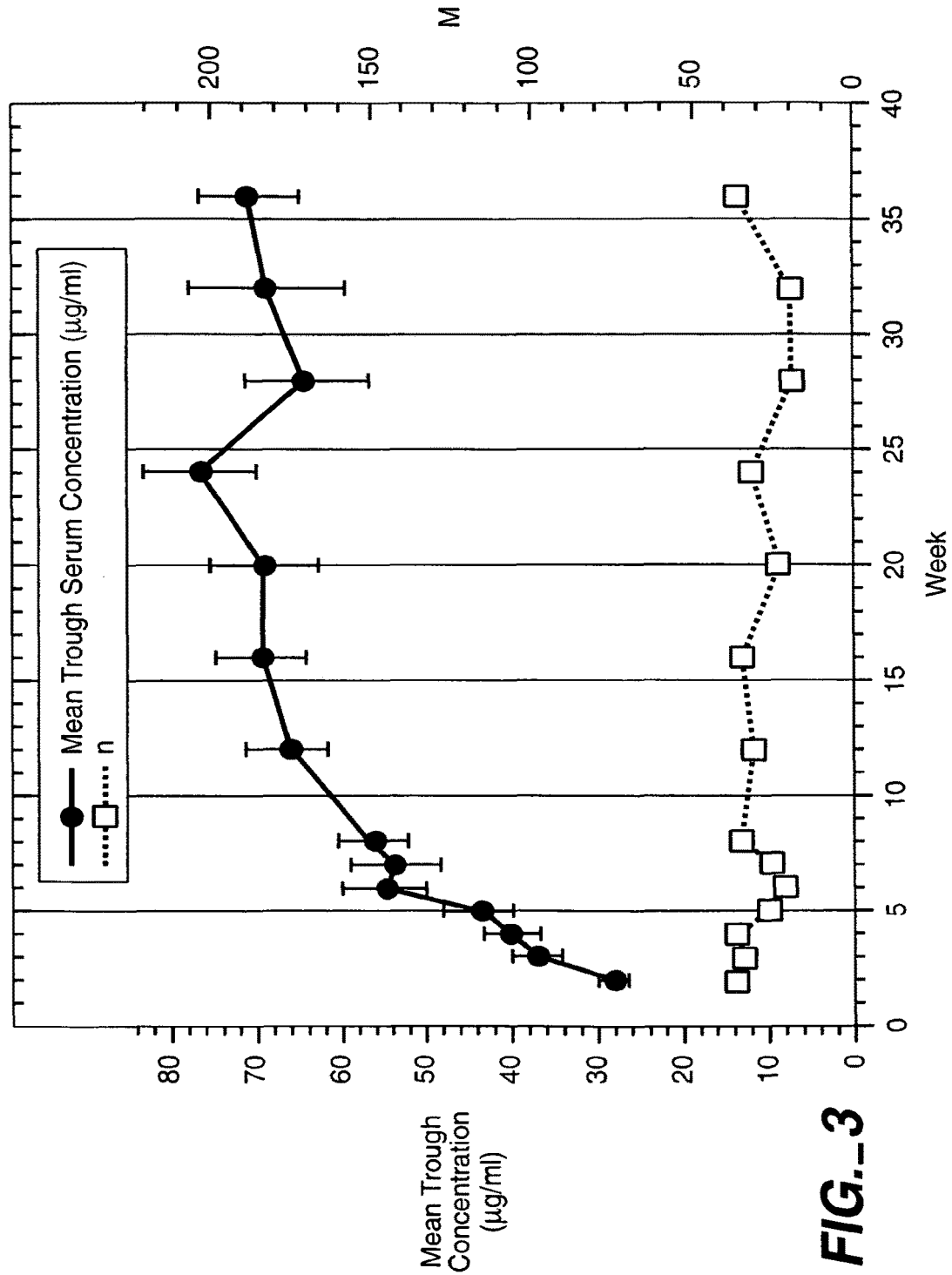


FIG. 3

U.S. Patent

Dec. 25, 2018

Sheet 3 of 5

US 10,160,811 B2

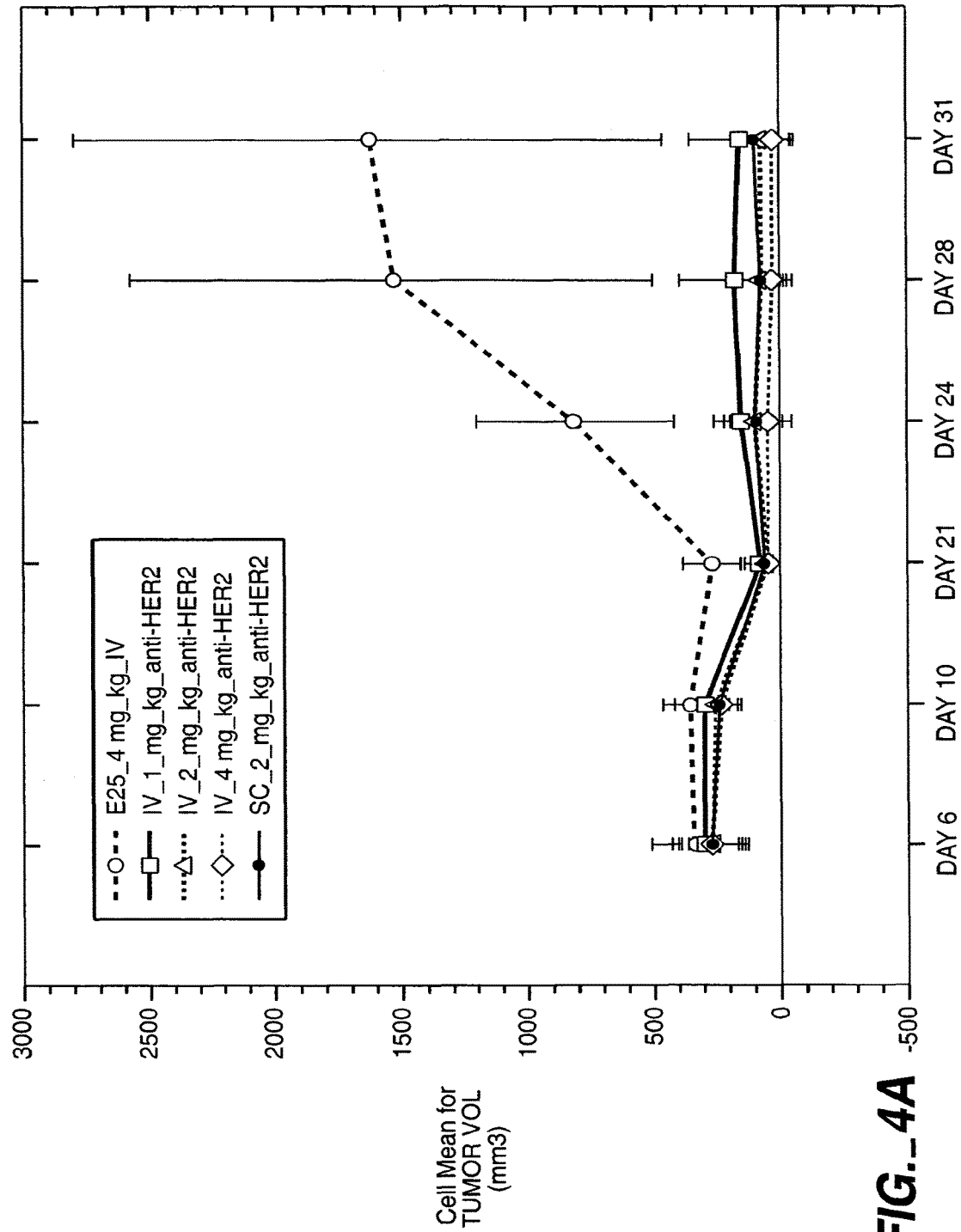


FIG. 4A

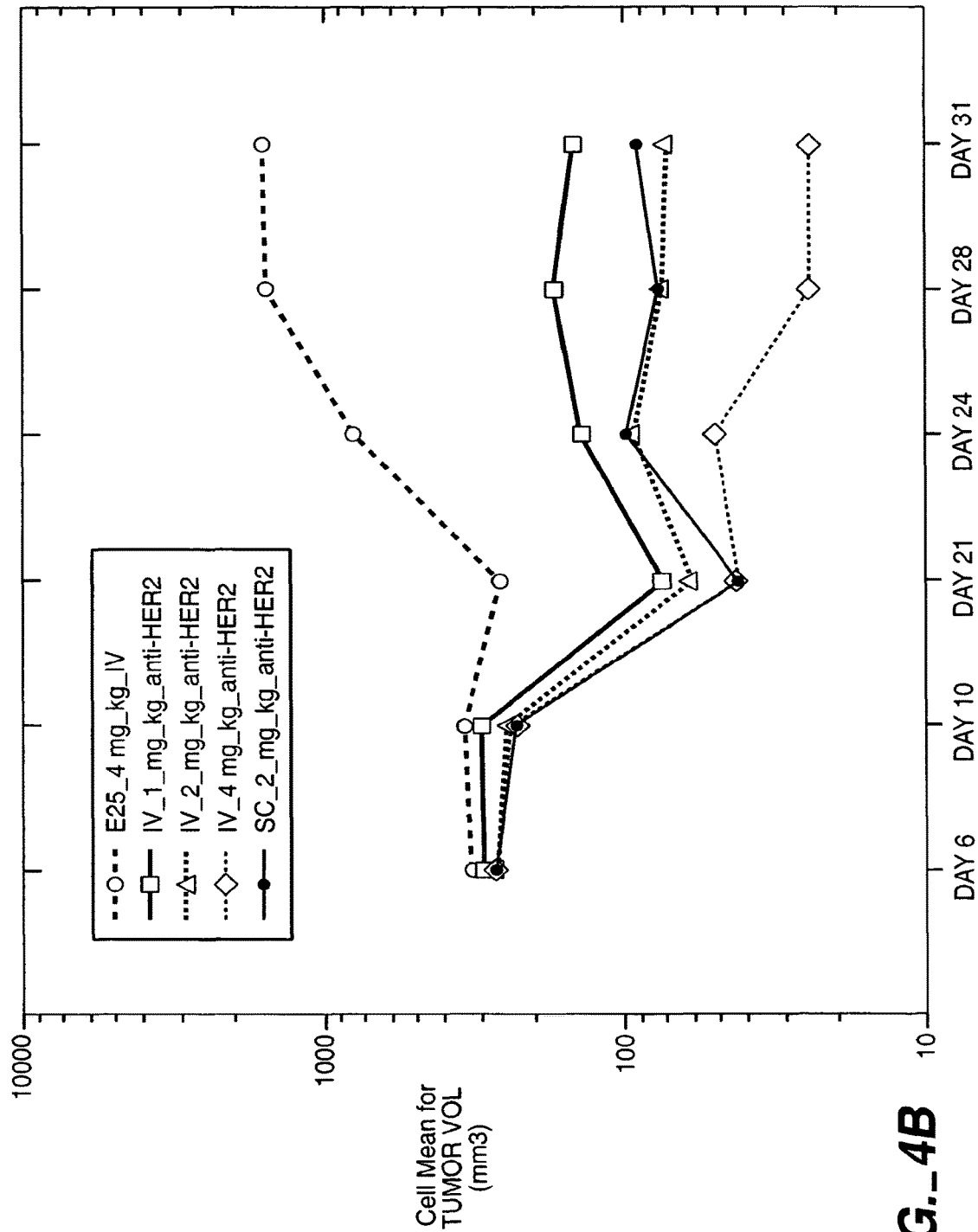


FIG. 4B

U.S. Patent

Dec. 25, 2018

Sheet 5 of 5

US 10,160,811 B2

VARIABLE LIGHT

	1	10	20	30	40
2C4	DTVMTQSHKIMSTSVGDRVSITC [KASQDV SIGVA----] WYQQR P				
	**	**** *	*		*
574	DIQMTQSPSSLSASVGDRVTITC [KASQDV SIGVA----] WYQQK P				
			* * ****		
hum kI	DIQMTQSPSSLSASVGDRVTITC [RASQSVSTSSYSYMH] WYQQK P				
		50	60	70	80
2C4	GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTF TISSVQA				
	**		* *	* *	*
574	GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTL TISSLQP				
		* ****			
hum kI	GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTL TISSLQP				
		90	100		
2C4	EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:10)				
	* *		* *		
574	EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:12)				

hum kI	EDFATYYC [QQYNSLPYT] FGQGTKVEIK (SEQ ID NO:14)				

FIG._5A

VARIABLE HEAVY

	1	10	20	30	40
2C4	EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS				
	**	** * * ****	**		*
574	EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA				
			** * *		
humIII	EVQLVESGGGSVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA				
		50	60	70	80
2C4	HGKSLEWIG [DVNPNSGGSIYNQRFGK] KASLTVD RSSRIVYM				
	* *	**		*** *	**** *
574	PGKGLEWVA [DVNPNSGGSIYNQRFGK] RFTLSVDR SKNTLYL				
		* *****	** *****	* * *	
humIII	PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDD SKNTLYL				
		90	100	110	
2C4	ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLVTSS (SEQ ID NO:11)				
	***	**		*	
574	QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGT LVTVSS (SEQ ID NO:13)				
		** ****			
humIII	QMNSLRAEDTAVYYCAR [GRGGGS--DY] WGQGT LVTVSS (SEQ ID NO:15)				

FIG._5B

US 10,160,811 B2

1

TREATMENT WITH ANTI-ERBB2
ANTIBODIES

RELATED APPLICATIONS

This application is divisional of U.S. Ser. No. 10/600,152 filed Jun. 20, 2003, which is a divisional of U.S. Ser. No. 09/648,067 filed Aug. 25, 2000 (now U.S. Pat. No. 6,627,196), which claims priority under 35 USC 119(e) to provisional application Nos. 60/151,018, filed Aug. 27, 1999 and 60/213,822, filed Jun. 23, 2000, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of disorders characterized by the overexpression of ErbB2 or disorders expressing epidermal growth factor receptor (EGFR), comprising administering to a human or animal presenting the disorders a therapeutically effective amount of an antibody that binds ErbB2. More specifically, the invention concerns the treatment of human patients susceptible to or diagnosed with cancer overexpressing ErbB2 or expressing EGFR, where the treatment is with an anti-ErbB2 antibody administered by front loading the dose of antibody during treatment by intravenous and/or subcutaneous administration. The invention optionally includes treatment of cancer in a human patient with a combination of an anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, a taxoid. The taxoid may be, but is not limited to paclitaxel or docetaxel. The invention further includes treatment of cancer in a human patient with a combination of anti-ErbB2 antibody and a chemotherapeutic agent, such as, but not limited to, an anthracycline derivative. Optionally, treatment with a combination of anti-ErbB2 and an anthracycline derivative includes treatment with an effective amount of a cardioprotectant. The present invention further concerns infrequent dosing of anti-ErbB2 antibodies.

BACKGROUND OF THE INVENTION

Proto-oncogenes that encode growth factors and growth factor receptors have been identified to play important roles in the pathogenesis of various human malignancies, including breast cancer. It has been found that the human ErbB2 gene (erbB2, also known as her2, or c-erbB-2), which encodes a 185-kd transmembrane glycoprotein receptor (p185^{HER2}) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human breast cancer (Slamon et al., *Science* 235:177-182 [1987]; Slamon et al., *Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells has been shown to cause their malignant transformation (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore et al., *Science* 237:78-182 [1987]). Transgenic mice that express HER2 were found to develop mammary tumors (Guy et al., *Proc. Natl. Acad. Sci. USA* 89:10578-10582 [1992]).

Antibodies directed against human erbB2 protein products and proteins encoded by the rat equivalent of the erbB2 gene (neu) have been described. Drebin et al., *Cell* 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat neu gene product. This antibody

2

with the neu proto-oncogene) and inhibits colony formation of these cells. In Drebin et al. *PNAS (USA)* 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of neu-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the neu oncogene was initially isolated) implanted into nude mice. Drebin et al. in *Oncogene* 2:387-394 (1988) discuss the production of a panel of antibodies against the rat neu gene product. All of the antibodies were found to exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate significant in vitro lysis of neu-transformed cells in the presence of complement, whereas none of the antibodies were able to mediate high levels of antibody-dependent cellular cytotoxicity (ADCC) of the neu-transformed cells. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions on the p185 molecule result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. Biological effects of anti-neu antibodies are reviewed in Myers et al., *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published Oct. 13, 1994. Hudziak et al., *Mol. Cell. Biol.* 9(3): 1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel, including 7C2 and 7F3, reduced cellular proliferation to a lesser extent in this assay. Hudziak et al. conclude that the effect of the 4D5 antibody on SKBR3 cells was cytostatic rather than cytotoxic, since SKBR3 cells resumed growth at a nearly normal rate following removal of the antibody from the medium. The antibody 4D5 was further found to sensitize p185^{erbB2}-overexpressing breast tumor cell lines to the cytotoxic effects of TNF- α . See also WO89/06692 published Jul. 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2): 979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); and D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994). Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses ErbB2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of ErbB2, and inhibit tumor cell growth in vitro.

McKenzie et al. *Oncogene* 4:543-548 (1989) generated a panel of anti-ErbB2 antibodies with varying epitope specificities, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of ErbB2 (see Maier et al. *Cancer Res.* 51:5361-5369 [1991]). Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the erbB2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells

US 10,160,811 B2

3

was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al. *PNAS (USA)* 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the erbB2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis in vitro via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al. *Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. of the preceding paragraphs. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth, whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus et al. saw a correlation between tumor inhibition in vivo and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu et al. *Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan. 6, 1994 and Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); and Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992).

A recombinant humanized anti-ErbB2 monoclonal antibody (a humanized version of the murine anti-ErbB2 antibody 4D5, referred to as rhuMAb HER2, HERCEPTIN®, or HERCEPTIN® anti-ErbB2 antibody) has been clinically active in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]). The recommended initial loading dose for HERCEPTIN® is 4 mg/kg administered as a 90-minute infusion. The recom-

4

mended weekly maintenance dose is 2 mg/kg and can be administered as a 30-minute infusion if the initial loading dose is well tolerated.

ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis, especially in patients with primary disease that involves axillary lymph nodes (Slamon et al., [1987] and [1989], supra; Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern, *Biochim Biophys Acta* 1198:165-184 [1994]), and has been linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga et al., *Oncology* 11(3 Suppl 1):43-48 [1997]). However, despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (Ibid). rhuMAb HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga et al., *Breast Cancer, Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

SUMMARY OF THE INVENTION

The present invention concerns the discovery that an early attainment of an efficacious target trough serum concentration by providing an initial dose or doses of anti-ErbB2 antibodies followed by subsequent doses of equal or smaller amounts of antibody (greater front loading) is more efficacious than conventional treatments. The efficacious target trough serum concentration is reached in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The target serum concentration is thereafter maintained by the administration of maintenance doses of equal or smaller amounts for the remainder of the treatment regimen or until suppression of disease symptoms is achieved.

The invention further concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of ErbB2 receptor comprising administering a therapeutically effective amount of an anti-ErbB2 antibody subcutaneously. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance to the anti-ErbB2 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable.

According to the invention, the method of treatment involves administration of an initial dose of anti-ErbB2 antibody of more than approximately 4 mg/kg, preferably more than approximately 5 mg/kg. The maximum initial dose or a subsequent dose does not exceed 50 mg/kg, preferably does not exceed 40 mg/kg, and more preferably does not exceed 30 mg/kg. Administration is by intravenous or subcutaneous administration, preferably intravenous infusion or bolus injection, or more preferably subcutaneous bolus injection. The initial dose may be one or more administrations of drug sufficient to reach the target trough serum concentration in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less.

According to the invention, the initial dose or doses is/are followed by subsequent doses of equal or smaller amounts

US 10,160,811 B2

5

of antibody at intervals sufficiently close to maintain the trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or subsequent dose does not exceed 50 mg/kg, and each subsequent dose is at least 0.01 mg/kg. Preferably the amount of drug administered is sufficient to maintain the target trough serum concentration such that the interval between administration cycles is at least one week. Preferably the trough serum concentration does not exceed 2500 µg/ml and does not fall below 0.01 µg/ml during treatment. The front loading drug treatment method of the invention has the advantage of increased efficacy by reaching a target serum drug concentration early in treatment. The subcutaneous delivery of maintenance doses according to the invention has the advantage of being convenient for the patient and health care professionals, reducing time and costs for drug treatment. Preferably, the initial dose (or the last dose within an initial dose series) is separated in time from the first subsequent dose by 4 weeks or less, preferably 3 weeks or less, more preferably 3 weeks or less, most preferably 1 week or less.

In an embodiment of the invention, the initial dose of anti-ErbB2 is 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous administration, such as intravenous infusion or subcutaneous bolus injection. The subsequent maintenance doses are 2 mg/kg delivered once per week by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated, the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In another embodiment, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In still another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

In yet another embodiment, the invention includes an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

In another embodiment, the invention includes initial doses of at least 1 mg/kg, preferably 4 mg/kg, anti-ErbB2 antibody on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

In another embodiment, the invention includes an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

In still another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. In one embodiment of the invention, each dose is approximately 25 mg/kg or less for a human patient, preferably approximately 10 mg/kg or less. This 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

In another embodiment, the invention includes a cycle of dosing in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The disorder preferably is a benign or malignant tumor characterized by the overexpression of the ErbB2 receptor, e.g. a cancer, such as, breast cancer, squamous cell cancer,

6

small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. The method of the invention may further comprise administration of a chemotherapeutic agent other than an anthracycline, e.g. doxorubicin or epirubicin. The chemotherapeutic agent preferably is a taxoid, such as TAXOL® (paclitaxel) or a TAXOL® derivative.

Preferred anti-ErbB2 antibodies bind the extracellular domain of the ErbB2 receptor, and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5, most preferably in a humanized form. Other preferred ErbB2-binding antibodies include, but are not limited to, antibodies 7C2, 7F3, and 2C4, preferably in a humanized form.

The method of the present invention is particularly suitable for the treatment of breast or ovarian cancer, characterized by the overexpression of the ErbB2 receptor.

The present application also provides a method of therapy involving infrequent dosing of an anti-ErbB2 antibody. In particular, the invention provides a method for the treatment of cancer (e.g. cancer characterized by overexpression of the ErbB2 receptor) in a human patient comprising administering to the patient a first dose of an anti-ErbB2 antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). For instance, the antibody may be administered about every three weeks, about two to about 20 times, e.g. about six times. The first dose and subsequent dose may each be from about 2 mg/kg to about 16 mg/kg; e.g. from about 4 mg/kg to about 12 mg/kg; and optionally from about 6 mg/kg to about 12 mg/kg. Generally, two or more subsequent doses (e.g. from about two to about ten subsequent doses) of the antibody are administered to the patient, and those subsequent doses are preferably separated from each other in time by at least about two weeks (e.g. from about two weeks to about two months), and optionally at least about three weeks (e.g. from about three weeks to about six weeks). The two or more subsequent doses may each be from about 2 mg/kg to about 16 mg/kg; or from about 4 mg/kg to about 12 mg/kg; or from about 6 mg/kg to about 12 mg/kg. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, and a package insert containing instructions to administer the antibody according to such methods.

The presently described dosing protocols may be applied to other anti-ErbB antibodies such as anti-epidermal growth factor receptor (EGFR), anti-ErbB3 and anti-ErbB4 antibodies. Thus, the invention provides a method for the treatment of cancer in a human patient, comprising administering an effective amount of an anti-ErbB antibody to the human patient, the method comprising administering to the patient an initial dose of at least approximately 5 mg/kg of the anti-ErbB antibody; and administering to the patient a plurality of subsequent doses of the antibody in an amount that is approximately the same or less than the initial dose. Alternatively, or additionally, the invention pertains to a method for the treatment of cancer in a human patient

US 10,160,811 B2

7

comprising administering to the patient a first dose of an anti-ErbB antibody followed by at least one subsequent dose of the antibody, wherein the first dose and subsequent dose are separated from each other in time by at least about two weeks. The invention additionally provides an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB antibody, and a package insert containing instructions to administer the antibody according to such methods.

In another aspect, the invention concerns an article of manufacture, comprising a container, a composition within the container comprising an anti-ErbB2 antibody, optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB2 receptor, and a package insert containing instructions to avoid the use of anthracycline-type chemotherapeutics in combination with the composition. According to the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody at an initial dose of 5 mg/kg followed by the same or smaller subsequent dose or doses. In another embodiment of the invention, the package insert further includes instructions to administer the anti-ErbB2 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose, most preferably for all doses.

In a further aspect, the invention provides a method of treating ErbB2 expressing cancer in a human patient comprising administering to the patient effective amounts of an anti-ErbB2 antibody and a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is a taxoid including, but not limited to, paclitaxel and docetaxel. In another embodiment, the chemotherapeutic agent is an anthracycline derivative including, but not limited to, doxorubicin or epirubicin. In still another embodiment of the invention, treatment with an anti-ErbB2 antibody and an anthracycline derivative further includes administration of a cardioprotectant to the patient. In still another embodiment, an anthracycline derivative is not administered to the patient with the anti-ErbB2 antibody. One or more additional chemotherapeutic agents may also be administered to the patient. The cancer is preferably characterized by overexpression of ErbB2.

The invention further provides an article of manufacture comprising a container, a composition within the container comprising an anti-ErbB2 antibody and a package insert instructing the user of the composition to administer the anti-ErbB2 antibody composition and a chemotherapeutic agent to a patient. In another embodiment, the chemotherapeutic agent is other than an anthracycline, and is preferably a taxoid, such as TAXOL®. In still another embodiment, the chemotherapeutic agent is an anthracycline, including but not limited to, doxorubicin or epirubicin. In yet another embodiment, the chemotherapeutic agent is an anthracycline and the package insert further instructs the user to administer a cardioprotectant.

The methods and compositions of the invention comprise an anti-ErbB2 antibody and include a humanized anti-ErbB2 antibody. Thus, the invention further pertains to a composition comprising an antibody that binds ErbB2 and the use of the antibody for treating ErbB2 expressing cancer, e.g., ErbB2 overexpressing cancer, in a human. The invention also pertains to the use of the antibody for treating EGFR expressing cancer. Preferably the antibody is a monoclonal antibody 4D5, e.g., humanized 4D5 (and preferably huMAb4D5-8 (HERCEPTIN® anti-ErbB2 antibody); or monoclonal antibody 2C4, e.g., humanized 2C4. The anti-

8

body may be an intact antibody (e.g., an intact IgG₁ antibody) or an antibody fragment (e.g., a Fab, F(ab')₂, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-ErbB2 antibody 2C4 are shown in FIGS. 5A and 5B.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura et al. *J. of Virology* 67(10):6179-6191 [October 1993]; Renz et al. *J. Cell Biol.* 125(6):1395-1406 [June 1994]). The anti-proliferative MAbs 4D5 and 3H4 bind adjacent to the transmembrane domain. The various ErbB2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The ErbB2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293S cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μ Ci each of ³⁵S methionine and ³⁵S cysteine. Supernatants were harvested either the ErbB2 MAbs or control antibodies were added to the supernatant and incubated 2-4 hours at 4° C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. SEQ ID NOs:8 and 9 depict the 3H4 and 4D5 epitopes, respectively.

FIG. 2 depicts with underlining the amino acid sequence of Domain 1 of ErbB2 (SEQ ID NO:1). Bold amino acids indicate the location of the epitope recognized by MAbs 7C2 and 7F3 as determined by deletion mapping, i.e. the "7C2/7F3 epitope" (SEQ ID NO:2).

FIG. 3 is a graph of anti-ErbB2 antibody (HERCEPTIN®) trough serum concentration (μ g/ml, mean \pm SE, dark circles) by week from week 2 through week 36 for ErbB2 overexpressing patients treated with HERCEPTIN® anti-ErbB2 antibody at 4 mg/kg initial dose, followed by 2 mg/kg weekly. The number of patients at each time point is represented by "n" (white squares).

FIG. 4A is a linear plot of tumor volume changes over time in mice treated with HERCEPTIN® anti-ErbB2 antibody. FIG. 4B is a semi-logarithmic plot of the same data as in FIG. 4A such that the variation in tumor volume for the treated animals is observed more readily.

FIGS. 5A and 5B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. 5A) and variable heavy (V_H) (FIG. 5B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 10 and 11, respectively); V_L and V_H domains of humanized Fab version 574 (SEQ ID Nos. 12 and 13, respectively), and human V_L and V_H consensus frameworks (hum κ 1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 14 and 15, respectively). Asterisks identify differences between humanized Fab version 574 and murine monoclonal antibody 2C4 or between humanized Fab version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (e.g.

substituted as follows—IleL2Thr; ArgL54Leu; TyrL55Glu; ThrL56Ser; AspH35Ser; and ValH48Ile) in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured in order to further improve or refine its affinity and/or other biological activities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

An “ErbB receptor” is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3 and ErbB4 receptors as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms “ErbB1”, “epidermal growth factor receptor” and “EGFR” are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including variants thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.).

“ErbB3” and “HER3” refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193-9197 (1989), including variants thereof. Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof.

The terms “ErbB4” and “HER4” herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and Plowman et al., *Nature*, 366:473-475 (1993), including variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

The terms “HER2”, “ErbB2” “c-Erb-B2” are used interchangeably. Unless indicated otherwise, the terms “ErbB2” “c-Erb-B2” and “HER2” when used herein refer to the human protein, and “erbB2,” “c-erb-B2,” and “her2” refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The “epitope 4D5” is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring

Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see FIG. 1) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

The “epitope 3H4” is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope is shown in FIG. 1, and includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The “epitope 7C2/7F3” is the region at the N-terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (i.e. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; SEQ ID NO:2).

The term “induces cell death” or “capable of inducing cell death” refers to the ability of the antibody to make a viable cell become nonviable. The “cell” here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which “overexpresses” ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cyto-technology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the “PI uptake assay in BT474 cells”.

The phrase “induces apoptosis” or “capable of inducing apoptosis” refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the ErbB2 receptor. Preferably the “cell” is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell may be a SKBR3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is

US 10,160,811 B2

11

one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an "annexin binding assay using BT474 cells" (see below).

Sometimes the pro-apoptotic antibody will be one which blocks HRG binding/activation of the ErbB2/ErbB3 complex (e.g. 7F3 antibody). In other situations, the antibody is one which does not significantly block activation of the ErbB2/ErbB3 receptor complex by HRG (e.g. 7C2). Further, the antibody may be one like 7C2 which, while inducing apoptosis, does not induce a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control).

The antibody of interest may be one like 7C2 which binds specifically to human ErbB2 and does not significantly cross-react with other proteins such as those encoded by the *erbB1*, *erbB3* and/or *erbB4* genes. Sometimes, the antibody may not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984). In such embodiments, the extent of binding of the antibody to these proteins (e.g., cell surface binding to endogenous receptor) will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science*, 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.*, 14(3):1909-1919 (1994); and Marchionni et al., *Nature*, 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. HRG β _{1,77-244}).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the

12

variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *NIH Publ. No.* 91-3242, Vol. I, pages 647-669 [1991]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different

US 10,160,811 B2

13

classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term “antibody” is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

“Antibody fragments” comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.* 8(10):1057-1062 [1995]); single-chain antibody molecules; and multi-specific antibodies formed from antibody fragments.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The “monoclonal antibodies” may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include “chimeric” antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 [1984]).

“Humanized” forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In

14

some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

“Single-chain Fv” or “sFv” antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term “diabodies” refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

As used herein, the term “salvage receptor binding epitope” refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

“Treatment” refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of

US 10,160,811 B2

15

treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the anti-ErbB2 antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP), or determining the response rates (RR) (see Example 1, below). Therapeutically effective amount also refers to a target serum concentration, such as a trough serum concentration, that has been shown to be effective in suppressing disease symptoms when maintained for a period of time.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I^{131} , I^{125} , Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXANTM); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylenelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabacin,

16

caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguanzone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK[®]; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL[®], Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE[®], Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL[®], and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders:

US 10,160,811 B2

17

Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

"Doxorubicin" is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2, 3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5, 12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and - β ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF- β ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF- α or TNF- β ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above:

By "solid phase" is meant a non-aqueous matrix to which the antibodies used in accordance with the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid

18

phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum HERCEPTIN® anti-ErbB2 antibody concentration" refers to the concentration of a drug, such as HERCEPTIN® anti-ErbB2 antibody, in the blood serum of an animal or human patient being treated with the drug. Serum concentration of HERCEPTIN® anti-ErbB2 antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is an ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery

US 10,160,811 B2

19

from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human patient, preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration. According to the present invention, front loading is achieved by an initial dose or doses delivered over three weeks or less that causes the animal's or patient's serum concentration to reach a target serum trough concentration. Preferably, the initial front loading dose or series of doses is administered in two weeks or less, more preferably in 1 week or less, including 1 day or less. Most preferably, where the initial dose is a single dose and is not followed by a subsequent maintenance dose for at least 1 week, the initial dose is administered in 1 day or less. Where the initial dose is a series of doses, each dose is separated by at least 3 hours, but not more than 3 weeks or less, preferably 2 weeks or less, more preferably 1 week or less, most preferably 1 day or less. To avoid adverse immune reaction to an antibody drug such as an anti-ErbB2 antibody (e.g., HERCEPTIN® anti-ErbB2 antibody) in an animal or patient who has not previously been treated with the antibody, it may be preferable to deliver initial doses of the antibody by intravenous infusion. The present invention includes front loading drug delivery of initial and maintenance doses by infusion or bolus administration, intravenously or subcutaneously.

Published information related to anti-ErbB2 antibodies includes the following issued patents and published applications: PCT/US89/00051, published Jan. 5, 1989; PCT/US90/02697, published May 18, 1990; EU 0474727 issued Jul. 23, 1997; DE 69031120.6, issued Jul. 23, 1997; PCT/US97/18385, published Oct. 9, 1997; SA 97/9185, issued Oct. 14, 1997; U.S. Pat. No. 5,677,171, issued Oct. 14, 1997; U.S. Pat. No. 5,720,937, issued Feb. 24, 1998; U.S. Pat. No. 5,720,954, issued Feb. 24, 1998; U.S. Pat. No. 5,725,856, issued Mar. 10, 1998; U.S. Pat. No. 5,770,195, issued Jun. 23, 1998; U.S. Pat. No. 5,772,997, issued Jun. 30, 1998; PCT/US98/2626, published Dec. 10, 1998; and PCT/US99/06673, published Mar. 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

II. Production of Anti-ErbB2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the

20

present invention. The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., *PNAS (USA)* 88:8691-8695 [1991]) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with $\frac{1}{5}$ to $\frac{1}{10}$ the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture

US 10,160,811 B2

21

medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio-*

22

Technology, 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and Human Antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer

programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 [1991]).

(iv) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science*, 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 [1992]). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, e.g. the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

US 10,160,811 B2

25

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')₂ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

(vi) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

26

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Rockville, Md.]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10⁶ per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca²⁺ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca²⁺ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FITC) (1 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 µg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art.

To identify anti-ErbB2 antibodies which inhibit growth of SKBR3 cells in cell culture by 50-100%, the SKBR3 assay described in WO 89/06692 can be performed. According to this assay, SKBR3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine

US 10,160,811 B2

27

serum, glutamine and penicillin streptomycin. The SKBR3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 2.5 µg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SKBR3 cells by 50-100% are selected for combination with the apoptotic antibodies as desired.

(vii) Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp. Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *momordica charantia* inhibitor, curcumin, croton, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB2 antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y and ¹⁸⁶Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionuclide to the antibody. See WO 94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor

28

pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g. avidin) which is conjugated to a cytotoxic agent (e.g. a radionuclide).

(ix) Immunoliposomes

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al., *J. National Cancer Inst.* 81(19):1484 (1989).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Pat. No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratio protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-ErbB2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins

comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature*, 312: 604-608 [1984]).

(xi) Antibody-Salvage Receptor Binding Epitope Fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (e.g. by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, e.g., by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased in vivo half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer in vivo half-life than that of the original antibody. If the antibody variant does not have a longer in vivo half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer in vivo half-life, and this process is continued until a molecule is obtained that exhibits a longer in vivo half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, e.g., on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence (5' to 3'): PKNS-SMISNTP (SEQ ID NO:3), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:4), HQNLSDGK (SEQ ID NO:5), HQNIS-DGK (SEQ ID NO:6), or VISSLGQ (SEQ ID NO:7), particularly where the antibody fragment is a Fab or F(ab)₂. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s) (5' to 3'): HQNLSDGK (SEQ ID NO:5), HQNISDGK (SEQ ID NO:6), or VISSLGQ (SEQ ID NO:7) and the sequence: PKNSSMISNTP (SEQ ID NO:3).

(xii) Purification of Anti-ErbB2 Antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced

intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

III. Determination of Anti-ErbB2 Antibody Concentration in Serum

The following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific rhuMAb HER2 (humanized anti-p185^{HER2} monoclonal antibody, including HERCEPTIN® anti-ErbB2 antibody) in a body fluid of a mammal including, but not limited to, serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids, and ocular vitreous gel.

Plate Binding Activity Assay for rhuMAb HER2 (Humanized Anti-p185^{HER2} Monoclonal Antibody)

The method of assaying rhuMAb HER2 described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of rhuMAb HER2 (Genentech, Inc., South San Francisco, Calif.), controls, and serum samples were diluted with Assay Diluent (PBS/0.5% BSA/0.05% Polysorbate 20/0.01% Thimerosal). The dilu-

US 10,160,811 B2

31

tions of standardized rhuMAb HER2 were prepared to span a range of concentrations useful for a standard curve. The samples were diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (recombinant p185^{HER2} (Genentech, Inc.) in 0.05 M sodium carbonate buffer) was added to each well of a microtiter plate and incubated at 2-8° C. for 12-72 hours. The coating solution was removed and each well was washed six times with water, then blotted to remove excess water:

An aliquot of Assay Diluent was added to each well and incubated for 1-2 hours at ambient temperature with agitation. The wells were washed as in the previous step.

Aliquots of diluted standard, control and sample solutions were added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish peroxidase; Organon Teknika catalog #55253 or equivalent) was diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution was added to each well and incubated at ambient temperature for 1 hour with agitation. The wells were washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in 12.5 ml 4 mM H₂O₂ in PBS) was added to each well and incubated for a sufficient period of time (approximately 8-10 minutes) in the dark at ambient temperature to allow color development. The reaction was stopped with an aliquot of 4.5 N sulfuric acid. Optical density was read at 490-492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

IV. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyltrimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURON-ICSTM or polyethylene glycol (PEG). Preferred lyophilized

32

anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial growth factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

V. Treatment with the Anti-ErbB2 Antibodies

It is contemplated that, according to the present invention, the anti-ErbB2 antibodies may be used to treat various conditions characterized by overexpression and/or activation of the ErbB2 receptor. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such

US 10,160,811 B2

33

as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The antibodies of the invention are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

The treatment of the present invention involves the administration of an anti-ErbB2 antibody to an animal or human patient, followed at intervals by subsequent doses of equal or smaller doses such that a target serum concentration is achieved and maintained during treatment. Preferably, maintenance doses are delivered by bolus delivery, preferably by subcutaneous bolus administration, making treatment convenient and cost-effective for the patient and health care professionals.

Where combined administration of a chemotherapeutic agent (other than an anticycline) is desired, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the EGFR, ErbB3, ErbB4, or vascular endothelial growth factor (VEGF). Alternatively, or additionally, two or more anti-ErbB2 antibodies may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient. The ErbB2 antibody may be co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by the ErbB2 antibody. However, simultaneous administration, or administration of the ErbB2 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and anti-ErbB2 antibody.

In addition to the above therapeutic regimens, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of anti-ErbB2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Where the treatment involves a series of

34

treatments, the initial dose or initial doses are followed at daily or weekly intervals by maintenance doses. Each maintenance dose provides the same or a smaller amount of antibody compared to the amount of antibody administered in the initial dose or doses.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays.

According to the invention, dosage regimens may include an initial dose of anti-ErbB2 of 6 mg/kg, 8 mg/kg, or 12 mg/kg delivered by intravenous or subcutaneous infusion, followed by subsequent weekly maintenance doses of 2 mg/kg by intravenous infusion, intravenous bolus injection, subcutaneous infusion, or subcutaneous bolus injection. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

Alternatively, the invention includes an initial dose of 12 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

Another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by 6 mg/kg once per 3 weeks.

Still another dosage regimen involves an initial dose of 8 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 8 mg/kg once per week or 8 mg/kg once every 2 to 3 weeks.

As an alternative regimen, initial doses of 4 mg/kg anti-ErbB2 antibody may be administered on each of days 1, 2 and 3, followed by subsequent maintenance doses of 6 mg/kg once per 3 weeks.

An additional regimen involves an initial dose of 4 mg/kg anti-ErbB2 antibody, followed by subsequent maintenance doses of 2 mg/kg twice per week, wherein the maintenance doses are separated by 3 days.

Alternatively, the invention may include a cycle of dosing in which delivery of anti-ErbB2 antibody is 2-3 times per week for 3 weeks. The 3 week cycle is preferably repeated as necessary to achieve suppression of disease symptoms.

The invention further includes a cyclic dosage regimen in which delivery of anti-ErbB2 antibody is daily for 5 days. According to the invention, the cycle is preferably repeated as necessary to achieve suppression of disease symptoms. Further information about suitable dosages is provided in the Examples below.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection

US 10,160,811 B2

35

needle). At least one active agent in the composition is an anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may comprise a package inserts with instructions for use, including, e.g., a warning that the composition is not to be used in combination with anthracycline-type chemotherapeutic agent, e.g. doxorubicin or epirubicin.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLES

Example 1: Preparation and Efficacy of HERCEPTIN® Anti-ErbB2 Antibody

Materials and Methods

Anti-ErbB2 Monoclonal Antibody

The anti-ErbB2 IgG₁κ murine monoclonal antibody 4D5, specific for the extracellular domain of ErbB2, was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990) and WO89/06692. Briefly, NIH 3T3/HER2-3₄₀₀ cells (expressing approximately 1×10⁵ ErbB2 molecules/cell) produced as described in Hudziak, et al., *Proc. Natl. Acad. Sci. (USA)* 84:7159 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10⁷ cells in 0.5 ml PBS on weeks, 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ³²P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, N.C.), was used as an isotype-matched control.

The treatment was performed with a humanized version of the murine 4D5 antibody (HERCEPTIN® anti-ErbB2 antibody). The humanized antibody was engineered by inserting the complementarity determining regions of the murine 4D5 antibody into the framework of a consensus human immunoglobulin IgG₁ (IgG₁) (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-ErbB2 monoclonal antibody has high affinity for p185^{HER2} (Dissociation constant [K_d]=0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of

36

p185^{HER2}, induces antibody-dependent cellular cytotoxicity (ADCC), and has been found clinically active, as a single agent, in patients with ErbB2-overexpressing metastatic breast cancers that had received extensive prior therapy. HERCEPTIN® anti-ErbB2 antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Eligibility Criteria

Patients had to fulfill all of the following criteria to be eligible for study admission:

Metastatic breast cancer

Overexpression of the ErbB2 (HER2) oncogene (2+ to 3+ as determined by immunohistochemistry or fluorescence in situ hybridization (FISH). [Tumor expression of ErbB2 can be determined by immunohistochemical analysis, as previously described (Slamon et al., [1987] and [1989], supra), of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used is murine 4D5 MAb, which has the same CDRs as the humanized antibody used for the treatment. Tumors are considered to over-express ErbB2 if at least 25% of tumor cells exhibit characteristic membrane staining for p185^{HER2}].

Bidimensionally measurable disease (including lytic bone lesions) by radiographic means, physical examination, or photographs

Measurable disease was defined as any mass reproducibly measurable in two perpendicular diameters by physical examination, X-ray (plain films), computerized tomography (CT), magnetic resonance imaging (MRI), ultrasound, or photographs.

Osteoblastic metastases, pleural effusions, or ascites were not considered to be measurable. Measurable lesions must be at least 1 cm in greatest dimension. Enumeration of evaluable sites of metastatic disease and number of lesions in an evaluable site (e.g. lung) had to be recorded on the appropriate Case Report Form (CRF). If a large number of pulmonary or hepatic lesions were present, the six largest lesions per site were followed.

The ability to understand and willingness to sign a written informed consent form

Women ≤18 years

Suitable candidates for receiving concomitant cytotoxic chemotherapy as evidenced by screening laboratory assessments of hematologic, renal, hepatic, and metabolic functions.

Exclusion Criteria

Patients with any of the following were excluded from study entry:

Prior cytotoxic chemotherapy for metastatic breast cancer
Patients may have received prior hormonal therapy (e.g. tamoxifen) for metastatic disease or cytotoxic therapy in the adjuvant setting.

Concomitant malignancy that has not been curatively treated

A performance status of <60% on the Karnofsky scale
Pregnant or nursing women; women of childbearing potential, unless using effective contraception as determined by the investigator

US 10,160,811 B2

37

Bilateral breast cancer (either both primary tumors must have 2+ to 3+ HER2 overexpression, or the metastatic site must have 2+ to 3+ HER2 overexpression)

Use of investigational or unlicensed agents within 30 days prior to study entry

Clinically unstable or untreated metastases to the brain (e.g. requiring radiation therapy)

Based upon the foregoing criteria, 469 patients were chosen, and enrolled in the study. Half the patients (stratified by chemotherapy) were randomized to additionally receive the HERCEPTIN® anti-ErbB2 antibody (see below).

Administration and Dosage

Anti-ErbB2 Antibody

On day 0, a 4 mg/kg dose of humanized anti-ErbB2 antibody (HERCEPTIN®, H) was administered intravenously, over a 90-minute period. Beginning on day 7, patients received weekly administration of 2 mg/kg antibody (i.v.) over a 90-minute period.

Chemotherapy

The patients received one of two chemotherapy regimens for a minimum of six cycles, provided their disease was not progressing: a) cyclophosphamide and doxorubicin or epirubicin (AC), if patients have not received anthracycline therapy in the adjuvant setting, or b) paclitaxel (T, TAXOL®), if patients have received any anthracycline therapy in the adjuvant setting. The initial dose of the HERCEPTIN® anti-ErbB2 antibody preceded the first cycle of either chemotherapy regimen by 24 hours. Subsequent doses of the antibody were given immediately before chemotherapy administration, if the initial dose of the antibody was well tolerated. If the first dose of the antibody was not well tolerated, subsequent infusions continued to precede chemotherapy administration by 24 hours. Patients were permitted to continue receiving chemotherapy beyond six cycles if, in the opinion of the treating physician, they were continuing to receive treatment benefit.

Cyclophosphamide (600 mg/m²) was given either by iv push over a minimum period of 3 minutes or by infusion over a maximum period of 2 hours.

Doxorubicin (60 mg/m²) or epirubicin (75 mg/m²) were given either by slow iv push over a minimum period of 3-5 minutes or by infusion over a maximum period of 2 hours, according to institutional protocol.

Paclitaxel (TAXOL®) was given at a dose of 175 mg/m² over 3 hours by intravenous administration. All patients receiving paclitaxel were premedicated with dexamethasone (or its equivalent) 20 mg×2, administered orally 12 and 6 hours prior to paclitaxel; diphenhydramine (or its equivalent) 50 mg, iv, administered 30 minutes prior to paclitaxel, and dimetidine (or another H₂ blocker) 300 mg, iv, administered 30 minutes prior to paclitaxel.

Response Criteria

Progressive Disease

Objective evidence of an increase of 25% or more in any measurable lesion. Progressive disease also includes those instances when new lesions have appeared. For bone lesions, progression is defined as a 25% increase in objective measurement by plain film, CT, MRI; symptomatic new lesions not due to fracture; or requirement for palliative radiotherapy.

Complete Response

Disappearance of all radiographically and/or visually apparent tumor for a minimum of 4 weeks. Skin and chest wall complete responses had to be confirmed by biopsy.

Partial Response

A reduction of at least 50% in the sum of the products of the perpendicular diameters of all measurable lesions for a

38

minimum period of 4 weeks. No new lesions may have appeared, nor may any lesions have progressed in size.

Minor Response

A reduction of 25% to 49% in the sum of the products of the perpendicular diameters of all measurable lesions. No new lesions may have appeared, nor may any lesions have progressed in size.

Stable Disease

No change of greater than 25% in the size of measurable lesions. No lesions may have appeared.

Time to disease progression (TTP) was calculated from the beginning of therapy to progression.

Confidence limits for response rates were calculated using the exact method for a single proportion. (Fleiss, J L, *Statistical Methods for Rates and Proportions* (ed. 2), New York, N.Y., Wiley, 1981, pp 13-17).

Results

At a median follow-up of 10.5 months, assessments of time to disease progression (TTP in months) and response rates (RR) showed a significant augmentation of the chemotherapeutic effect by HERCEPTIN® anti-ErbB2 antibody, without increase in overall severe adverse events (AE):

TABLE 1

HERCEPTIN® Anti-ErbB2 Antibody Efficacy				
	Enrolled	TTP (months)	RR (%)	AE (%)
CRx	234	5.5	36.2	66
CRx + H	235	8.6*	62.00**	69
AC	145	6.5	42.1	71
AC + H	146	9.0	64.9	68
T	89	4.2	25.0	59
T + H	89	7.1	57.3	70

*p < 0.001 by log-rank test;

**p < 0.01 by X² test;

CRx: chemotherapy;

AC: anthracycline/cyclophosphamide treatment;

H: HERCEPTIN® anti-ErbB2 antibody;

T: TAXOL®

A syndrome of myocardial dysfunction similar to that observed with anthracyclines was reported more commonly with a combined treatment of AC+H (18% Grade 3/4) than with AC alone (3%), T (0%), or T+H (2%).

These data indicate that the combination of anti-ErbB2 antibody treatment with chemotherapy markedly increases the clinical benefit, as assessed by response rates and the evaluation of disease progression. However, due to the increased cardiac side-effects of doxorubicin or epirubicin, the combined use of anthracyclines with anti-ErbB2 antibody therapy is contraindicated. The results, taking into account risk and benefit, favor treatment with HERCEPTIN® anti-ErbB2 antibody and paclitaxel (TAXOL®) where a combined treatment regimen is desired.

Example 2: Pharmacokinetic and Pharmacodynamic Properties of Anti-ErbB2 Antibody (HERCEPTIN®)

HERCEPTIN® anti-ErbB2 antibody was administered by intravenous infusion to human patients selected according to the criteria provided in Example 1. An initial dose of 4 mg/kg HERCEPTIN® anti-ErbB2 antibody was delivered by intravenous infusion, followed by subsequent i.v. infusions of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody weekly for several weeks. Two hundred thirteen patients began this treatment regimen and serum drug concentration was obtained beyond 8 weeks for fewer than 90 patients as

selective discontinuation of patients with rapidly progressing disease occurred. Of the 213 patients who began treatment, serum trough concentration data were available for 80 patients at Week 12, for 77 patients at Week 16, for 44 patients at Week 20, for 51 patients at Week 24, for 25 patients at Week 28, for 23 patients at Week 32, and for 37 patients at Week 36.

HERCEPTIN® Anti-ErbB2 Antibody Trough Serum Concentrations for Weeks 0-36

The HERCEPTIN® anti-ErbB2 antibody trough serum concentrations ($\mu\text{g/ml}$, mean \pm SE) from Week 2 through Week 36 are plotted in FIG. 3 (dark circles). The number of patients was fairly constant because data from patients discontinued from the program due to rapidly progressing disease were excluded from this analysis. Trough serum concentrations tended to increase through Week 12 and tended to plateau after that time.

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for Weeks 1-8

Some HERCEPTIN® anti-ErbB2 antibody serum concentration data were available for 212 of the original 213 patients. Trough and peak serum concentration data reflecting the first HERCEPTIN® anti-ErbB2 antibody infusion were available for 195 of the 212 patients. For the seventh infusion, trough serum concentration data were available for 137/212 patients and peak serum concentration data were available for 114/212 patients. Table 2 presents a summary of statistics from trough and peak serum concentrations for the first 8 weeks of treatment. Peak samples were drawn shortly after the end of HERCEPTIN® anti-ErbB2 antibody administration; trough samples were drawn prior to the subsequent dose (i.e., 1 week later). Serum concentrations of HERCEPTIN® anti-ErbB2 antibody were determined as disclosed herein.

TABLE 2

HERCEPTIN® Anti-ErbB2 Antibody Trough and Peak Serum Concentrations for the First 8 Weeks of Treatment ($\mu\text{g/ml}$)						
	Dose Number	n	Mean	SD	Minimum	Maximum
Peak	1	195	100.3	35.2	30.7	274.6
Trough		195	25.0	12.7	0.16	60.7
Peak	2	190	74.3	31.3	20.8	307.9
Trough		167	30.4	16.0	0.2	74.4
Peak	3	167	75.3	26.8	16.1	194.8
Trough		179	33.7	17.9	0.2	98.2
Peak	4	175	80.2	26.9	22.2	167
Trough		132	38.6	20.1	0.2	89.4
Peak	5	128	85.9	29.2	27.8	185.8
Trough		141	42.1	24.8	0.2	148.7
Peak	6	137	87.2	32.2	28.9	218.1
Trough		115	43.2	24.0	0.2	109.9
Peak	7	114	89.7	32.5	16.3	187.8
Trough		137	48.8	24.9	0.2	105.2
Peak	8	133	95.6	35.9	11.4	295.6

The data in Table 2 suggest that there was an increase in trough serum concentration over time. Of the many patients studied, there were 18 patients for whom the trough concentrations did not exceed 20 $\mu\text{g/ml}$ from Week 2 through Week 8. A HERCEPTIN® anti-ErbB2 antibody trough serum concentration of 20 $\mu\text{g/ml}$ was nominally targeted for these studies based on prior pharmacologic studies in animals and exploratory analyses in clinical trials.

Patient response status was evaluated relative to serum concentration of HERCEPTIN® anti-ErbB2 antibody. For this purpose, mean serum concentration (an average of troughs and peaks) was calculated for various times and patient response status (where the patient response status was determined by an independent Response Evaluation

Committee). The increase in serum concentration between Weeks 2 and 8 appeared to be greater in responders than in nonresponders, suggesting that there is a relationship between response status and HERCEPTIN® anti-ErbB2 antibody serum concentration. A statistical analysis (analysis of variance) of trough serum concentration values at Week 2 and an average of Weeks 7 and 8 in relation to response status indicated a highly significant relationship between response status and average trough of Weeks 7 and 8 ($p < 0.001$). The results indicated that there was a significant difference between the trough serum concentration (average troughs of Weeks 7 and 8) in the responders and nonresponders: trough concentrations were 60 ± 20 $\mu\text{g/ml}$ in the responders versus 44 ± 25 $\mu\text{g/ml}$ in the nonresponders (mean \pm SD). HER2 overexpression level and type of metastatic sites were associated with significant differences in trough serum concentrations. At Week 2, patients with 2+ HER2 overexpression had significantly higher trough serum concentrations ($n=40$, mean=28.8 $\mu\text{g/ml}$, SD=10.4) compared with patients with 3+ HER2 overexpression ($n=155$, mean=24.1 $\mu\text{g/ml}$, SD=13.1). This difference in the average trough serum concentrations for Weeks 7 and 8 was no longer statistically significant. Further, at Week 2, patients with superficial disease had significantly higher trough serum concentrations ($n=12$, mean 34.1 $\mu\text{g/ml}$, SD=12.0) compared with patients with visceral disease ($n=183$, mean=24.4 $\mu\text{g/ml}$, SD=12.6). This difference in the average trough serum concentrations for Weeks 7 and 8 was significant. These data indicate that the rise in trough serum concentrations between Weeks 2 and 7/8 occurs for human patients with various disease profiles.

In a subsequent, similarly designed study, human breast cancer patients were treated with a loading dose of 8 mg/kg followed by maintenance doses of 4 mg/kg weekly. The results of this preliminary human study indicated that an 8 mg/kg load:4 mg/kg weekly maintenance regimen was efficacious in reducing tumor volume in the patients.

The data disclosed in this Example indicate that front loading of antibody, such that a target serum concentration is reached more quickly, may be associated with improved outcomes.

Example 3: I.V. Bolus Delivery and Subcutaneous Infusion of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

The efficacy of infusion or bolus delivery of humanized anti-ErbB2 antibody (HERCEPTIN® see Example 1 for preparation), either by intravenous injection or subcutaneous injection, was examined. The purpose of the study was to ask whether subcutaneous delivery was feasible and whether the convenient subcutaneous bolus delivery was useful in treating metastatic breast cancer in animals inoculated with a cell line that overexpresses the HER2 gene. The results, detailed below, show that i.v. and s.c. infusion and bolus delivery are feasible treatment methodologies.

A study in a nude mouse xenograft model, which incorporates a human breast cancer cell line that naturally overexpresses the HER2 gene (BT-474M1, derived from BT-474 cells, ATCC Accession number HTB-20), comparing tumor volume as a function of i.v. bolus versus s.c. infusion was performed as follows. In the first study athymic nude nu nu 7-9 week old female mice were obtained from Taconic Inc (Germantown, N.Y.). To initiate tumor development, each mouse was inoculated subcutaneously with 3×10^6 BT474M1 cells suspended in Matrigel™. When tumor nodules reached a volume of approximately 100 mm³, animals were randomized to 4 treatment groups. The groups were treated according to Table 3.

US 10,160,811 B2

41

42

TABLE 3

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Infusion				
Group, Dose, Antibody	Target Serum Conc. µg/ml	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose
1—Control, rhuMab E25	20	IV LD and SC infusion	2.20	0.250 mg/ml (infusate)
2—Low Dose SC rhuMab HER2	1	IV LD and SC infusion	0.313	0.050 mg/ml (infusate)
3—High Dose SC rhuMab HER2	20	IV LD and SC infusion	6.25	1.00 mg/ml (infusate)
4—IV Multi-Dose rhuMab HER2	20 (trough)	IV LD and MD	4.00	2 mg/kg/week (IV bolus)

Serum Conc. = concentration in serum.

LD = loading dose.

MD = maintenance dose.

Infusate concentration was calculated to achieve targeted serum concentration using Alzet® osmotic minipumps (Alza Corp., Palo Alto, CA).

Animals were exposed to estrogen by subcutaneous sustained release estrogen pellet 9 days before the start of dosing to promote growth of grafted tumor cells. The animals were inoculated with the BT474M1 cells 8 days before the beginning of treatment and tumors were allowed to grow. The animals were then treated with nonrelevant antibody E25 (non-specific for HER2 receptor, but a member of the monoclonal IgG class) or test antibody HERCEPTIN® anti-ErbB2 antibody as indicated in Table 3. The dosage levels were selected to achieve target serum concentrations of HERCEPTIN®, either 1 µg/ml or 20 µg/ml, by subcutaneous pump infusion or by i.v. bolus delivery. The study groups were treated until day 35. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly (just prior to dosing for Group 4) using 3 mice/group/time point. The anti-ErbB2 antibody concentration was determined according to the method disclosed herein involving standard techniques. Tumor volumes were measured two days before dosing began and twice per week from day 6 to day 35 in the study for which data is tabulated below. Tumors were measured in three dimensions and volumes were expressed in mm³. Efficacy was determined by a statistical comparison (ANOVA) of tumor volumes of test animals relative to untreated control animals.

As shown in Table 4, below, treatment of the BT474M1 tumor-bearing mice with HERCEPTIN® anti-ErbB2 antibody by the indicated dosage methods significantly inhibited the growth of the tumors. All HERCEPTIN®-treated groups showed similar inhibition of tumor growth relative to the control group. No dose-response was observed.

The results tabulated above indicate that maintenance of a serum concentration of approximately 2 µg/ml was as effective as a concentration of 20 µg/ml in this study. The results indicated that dosing by subcutaneous infusion was as effective as intravenous bolus dosing and achieved similar trough serum concentrations. The results also indicate that the dose levels studied are at the top of the dose-response curve in this model and that subcutaneous dosing is effective in treating breast cancer tumors. Thus, subcutaneous administration of maintenance doses is feasible as part of a HERCEPTIN® anti-ErbB2 antibody treatment regimen.

Example 4: I.V. Bolus and Subcutaneous Bolus Deliveries of HERCEPTIN® Anti-ErbB2 Antibody Effectively Decrease Tumor Volume in the Mouse

Subcutaneous bolus delivery is convenient and cost-effective for the patient and health care professionals. The results of the study disclosed in this example indicate that subcutaneous bolus delivery was as effective as intravenous bolus delivery in reducing breast cell tumor size in a mouse.

This study was set up as disclosed herein in Example 3 for the comparison of intravenous bolus and subcutaneous infusion delivery. A sustained release estrogen implant was inserted subcutaneously one day before tumor cell inoculation as described in Example 3. Six days after tumor cell inoculation, the initial tumor measurement was performed. Seven days after tumor cell inoculation, the first dose of control antibody or HERCEPTIN® anti-ErbB2 antibody was delivered. The animal groups, type of delivery, loading dose and maintenance doses are provided in Table 4. Animals were dosed once weekly for 4 weeks.

TABLE 4

Comparison of S.C. Infusion and I.V. Bolus Delivery			
Treatment Group	Tumor Volume (mm ³), Day 35, (n = 14)	Tumor Volume (area under curve) Day 6-Day 35 (n = 13)	HERCEPTIN® Serum Conc. (µg/ml), Day 27, (n = 3)
control s.c. infusion	764 ± 700	5650 ± 4700	4.16 ± 1.94
s.c. infusion (low dose)	80.6 ± 158	1610 ± 1250	2.11 ± 1.74
s.c. infusion (high dose)	31 ± 75.6	1440 ± 1140	22.1 ± 5.43
i.v. bolus dose*	49.7 ± 95.7	2150 ± 1480	21.7 ± 17.1**

s.c. = subcutaneous delivery;

i.v. = intravenous delivery.

*4.0 mg/kg Loading Dose and 2.0 mg/kg/week Maintenance Dose.

**at predose (trough serum concentration immediately prior to a maintenance dose)

US 10,160,811 B2

43

TABLE 5

Animal Groups and Doses for Comparison of I.V. Bolus and S.C. Bolus Delivery				
Group	Route of Administration	Loading Dose (mg/kg)	Maintenance Dose (mg/kg/week)	n
1—Control rhuMAb E25	IV	8	4	10
2—rhuMAb HER2	IV	2	1	10
3—rhuMAb HER2	IV	4	2	10
4—rhuMAb HER2	IV	8	4	10
5—rhuMAb HER2	SC	4	2	10

IV = intravenous;

SC = subcutaneous;

n = number of animals per group.

The mice were treated according to the information in Table 4 and using the techniques disclosed in Example 3. The serum concentration of HERCEPTIN® anti-ErbB2 antibody was measured weekly before each weekly i.v. maintenance dose according to the procedure described

44

TABLE 6-continued

IV versus SC Bolus Delivery: Serum HERCEPTIN® Anti-ErbB2 Antibody Concentration Serum Concentration, µg/ml				
Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
4—rhu MAb HER2 (IV, 4 mg/kg)	0 (0)	29.6 (13.5)	37.7 (14.4)	46.2 (13.8)
5—rhu MAb HER2 (SC, 2 mg/kg)	0 (0)	12.5 (7.33)	16.9 (10.2)	17.6 (10.7)

n = 10 for time points Days 0, 7 and 14.

N = 9 for Day 21.

Table 7 shows the relative efficacy of intravenous bolus delivery and subcutaneous bolus delivery for Groups 1-5 having achieved the serum antibody concentrations presented in Table 6. For this study, efficacy was measured as a decrease in tumor volume. Tumor volume was measured twice weekly.

TABLE 7

Efficacy of HERCEPTIN® Anti-ErbB2 Antibody Measured as a Change in Tumor Volume Comparing Intravenous Bolus and Subcutaneous Bolus Delivery, Mean (SD)					
Treatment Group (Delivery, MD)	Tumor Vol. Day 6, mm ³	Tumor Vol. Day 28, mm ³	Tumor Vol. Day 31, mm ³	Day 6-Day 31* Area Under Curve Tumor Vol., mm ³	Tumor Growth Rate on Log (TM + 1)
1—IV Control	321 (190)	1530 (1040)	1630 (1170)	13600 (7230)	0.0660 (0.0200)
2—IV Herceptin 1 mg/kg	297 (130)	175 (215)	151 (188)	4690 (1400)	-0.0505 (0.142)
3—IV Herceptin 2 mg/kg	269 (129)	75.7 (92.4)	73.6 (84.5)	3510 (1220)	-0.0608 (0.110)
4—IV Herceptin 4 mg/kg	272 (117)	25.3 (75.9)	25.8 (72.9)	2880 (1230)	-0.0810 (0.0859)
5—SC Herceptin 2 mg/kg	268 (117)	76.2 (98.8)	90.4 (105)	3230 (1440)	-0.0304 (0.104)

N = 10 for each data point.

TM = tumor measurement.

IV = intravenous.

SC = subcutaneous.

MD = maintenance dose.

Tumor Vol. = tumor volume, mm³.

*Day 17 excluded due to measurement error.

Tumor growth rate calculated on Day 21-Day 31 Log(TM + 1). Area under the curve is the area beneath a plot of tumor volume versus time.

herein and using standard techniques. The control E25 antibody serum concentration was determined according to standard immunoassay techniques. Table 6 shows the increase in HERCEPTIN® anti-ErbB2 antibody serum concentrations with time.

TABLE 6

IV versus SC Bolus Delivery: Serum HERCEPTIN® Anti-ErbB2 Antibody Concentration Serum Concentration, µg/ml				
Treatment Group (delivery, MD)	Day 0 Mean (SD)	Day 7 Mean (SD)	Day 14 Mean (SD)	Day 21 Mean (SD)
1—Control rhu MAb E25 (IV, 4 mg/kg)	0 (0)	25.9 (8.29)	34.6 (11.2)	38.5 (14.4)
2—rhu MAb HER2 (IV, 1 mg/kg)	0 (0)	4.96 (3.79)	8.55 (5.83)	8.05 (4.67)
3—rhu MAb HER2 (IV, 2 mg/kg)	0 (0)	13.4 (9.24)	18.9 (12.0)	22.6 (9.21)

FIGS. 4A and 4B are graphical plots of changes in tumor volume over time, some of which data is found in Table 7. FIG. 4A is a linear plot of tumor volume versus time. FIG. 4B is a semilogarithmic plot of the same data, allowing the test points be viewed more clearly. The data in Table 7 and FIGS. 4A and 4B indicate that, although a dose-related response was not observed between HERCEPTIN-treated groups, dosing by subcutaneous bolus was as effective as intravenous bolus dosing and achieved similar trough serum concentrations.

Example 5: Regimens for Intravenous and Subcutaneous Delivery of Anti-ErbB2 Antibody

According to the invention, methods of anti-ErbB2 antibody (e.g., HERCEPTIN®) delivery comprise greater front loading of the drug to achieve a target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including one day or less. According to the inven-

US 10,160,811 B2

45

tion, this initial dosing is followed by dosing that maintains the target serum concentration by subsequent doses of equal or smaller amount. An advantage of the methods of the invention is that the maintenance dosing may be less frequent and/or delivered by subcutaneous injection, making the treatment regimens of the invention convenient and cost-effective for the patient and medical professionals administering the antibody. In addition, a subcutaneous maintenance dose regimen may be interrupted by intravenous dosing (such as infusion) when the patient's chemotherapy requires delivery of other drugs by intravenous injection.

To test the following dosage regimens, human subjects are selected according to the criteria disclosed in Example 1, above. The number of initial doses is one or more doses sufficient to achieve an efficacious target serum concentration in approximately 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, and most preferably 1 week or less, including 1 day or less. The number of maintenance doses may be one or more doses sufficient to achieve suppression of disease symptoms, such as a decrease in tumor volume. The maintenance doses are equal to or smaller than the initial dose or doses, consistent with an object of the invention of administering HERCEPTIN® anti-ErbB2 antibody by regimens providing greater front loading. The specific drug delivery regimens disclosed herein are representative of the invention and are not meant to be limiting.

In one trial, an initial dose of 6 mg/kg, 8 mg/kg, or 12 mg/kg of HERCEPTIN® anti-ErbB2 antibody is delivered to human patients by intravenous or subcutaneous injection. Initial doses (loading doses) are delivered by intravenous infusion or bolus injection or preferably subcutaneous bolus injection. Preferably a target trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 µg/ml¹ is achieved (averaged for all patients in the treatment group) and maintained by subsequent doses of anti-ErbB2 antibody that are equal to or smaller than the initial dose. In one method, a target trough serum concentration is achieved and maintained by once-per-week deliveries of 2 mg/kg HERCEPTIN® anti-ErbB2 antibody by intravenous or subcutaneous injection for at least eight weeks. Alternatively, for this or any dosage regimen disclosed herein, subcutaneous continuous infusion by subcutaneous pump is used to delivery subsequent maintenance doses.

In another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 µg/ml, averaged for an entire treatment group.

In another method, an initial (front loading) dose of 12 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous injection (infusion or bolus injection) or by subcutaneous bolus injection. This is followed by intravenous bolus injections, intravenous infusion, subcutaneous infusion, or subcutaneous bolus injection of 6 mg/kg at 3-week intervals to maintain a trough serum concentration of approximately 10-20 µg/ml.

In yet another method, an initial (front loading) dose of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody is delivered by intravenous infusion or bolus injection, or preferably by subcutaneous bolus injection or infusion. This is followed by

46

administration of 8 mg/kg per week or 8 mg/kg per 2-3 weeks to maintain a trough serum concentration of HERCEPTIN® anti-ErbB2 antibody of approximately 10-20 µg/ml. Maintenance doses are delivered by intravenous infusion or bolus injection, or preferably by subcutaneous infusion or bolus injection.

In another method, the front loading initial dose is a series of intravenous or subcutaneous injections, for example, one on each of days 1, 2, and 3 of at least 1 mg/kg for each injection (where the amount of anti-ErbB2 antibody delivered by the sum of initial injections is more than 4 mg/kg), followed by maintenance doses of 6 mg/kg once each 3 week interval to maintain a target trough serum concentration (for example, approximately 10-20 µg/ml) of HERCEPTIN® anti-ErbB2 antibody. The maintenance doses are delivered by intravenous infusion or bolus injection or by subcutaneous infusion or subcutaneous bolus injection.

In yet another method, the front loading is by intravenous infusion of at least 1 mg/kg, preferably 4 mg/kg on each of five consecutive days, followed by repeats of this cycle a sufficient number of times to achieve suppression of disease symptoms. Following the initial dose or doses, subsequent doses may be delivered by subcutaneous infusion or bolus injection if tolerated by the patient. Such subcutaneous delivery is convenient and cost-effective for the patient and administering health care professionals.

In still another method, HERCEPTIN® anti-ErbB2 antibody is delivered initially as at least 2 intravenous infusions per week for three weeks, followed by repeats of this cycle to maintain an efficacious trough serum concentration of HERCEPTIN® anti-ErbB2 antibody. The dose is at least 4 mg/kg of anti-ErbB2 antibody, preferably at least 5 mg/kg. The maintenance drug deliveries may be intravenous or subcutaneous.

Where the animal or patient tolerates the antibody during and after an initial dose, delivery of subsequent doses may be subcutaneous, thereby providing greater convenience and cost-effectiveness for the patient and health care professionals.

In animal studies, an initial dose of more than 4 mg/kg, preferably more than 5 mg/kg delivered by intravenous or subcutaneous injection, is followed by subcutaneous bolus injections of 2 mg/kg twice per week (separated by 3 days) to maintain a trough serum concentration of approximately 10-20 µg/ml. In addition, where the animal or patient is known to tolerate the antibody, an initial dose of HERCEPTIN® anti-ErbB2 antibody is optionally and preferably deliverable by subcutaneous bolus injection followed by subcutaneous maintenance injections.

While target serum concentrations are disclosed herein for the purpose of comparing animal studies and human trials, target serum concentrations in clinical uses may differ. The disclosure provided herein guides the user in selecting a front loading drug delivery regimen that provides an efficacious target trough serum concentration.

The methods of the invention disclosed herein optionally include the delivery of HERCEPTIN® anti-ErbB2 antibody in combination with a chemotherapeutic agent (other than an anthracycline derivative) to achieve suppression of disease symptoms. The chemotherapeutic agent may be delivered with HERCEPTIN® anti-ErbB2 antibody or separately and according to a different dosing schedule. For example, subcutaneous delivery of HERCEPTIN® anti-ErbB2 antibody with TAXOL® is included in the invention. In addition, intravenous or subcutaneous injection of 8 mg/kg HERCEPTIN® anti-ErbB2 antibody, followed by intravenous or subcutaneous injection of 6 mg/kg HERCEPTIN® anti-ErbB2 antibody every 3 weeks is administered in combination with a chemotherapeutic agent, such as a taxoid (e.g. paclitaxel 175 mg/m² every 3 weeks) or an anthracy-

US 10,160,811 B2

47

cline derivative (e.g. doxorubicin 60 mg/m² or epirubicin 75 mg/m² every 3 weeks). Optionally, where an anthracycline derivative is administered, a cardioprotectant (e.g. 600 mg/m² cyclophosphamide every 3 weeks) is also administered. In another combination therapy, anti-ErbB2 antibody is administered in a loading dose of more than 4 mg/kg, preferably more than 5 mg/kg, and more preferably at least 8 mg/kg. The loading dose is followed by maintenance doses of at least 2 mg/kg weekly, preferably 6 mg/kg every 3 weeks. The combination therapy includes administration of a taxoid during treatment with anti-ErbB2 antibody. According to one embodiment of the invention, the taxoid is paclitaxel and is administered at a dose of 70-100 mg/m²/week. According to another embodiment of the invention, the taxoid is docetaxel and is administered at a dose of 30-70 mg/m²/week.

Example 6: HERCEPTIN® Administered
Intravenously Every Three Weeks in Combination
with Paclitaxel

Currently, the recommended dose of HERCEPTIN® is 2 mg/kg once weekly. Patients will be administered HERCEPTIN® every three weeks instead of weekly, along with paclitaxel (175 mg/m² every three weeks). Simulation of the proposed treatment regimen suggests that the trough serum concentrations will be 17 mcg/ml, in the range (10-20 mcg/ml) of the targeted trough serum concentrations from previous HERCEPTIN® IV clinical trials. After the first 12 patients the PK parameters will be assessed, if exposure is felt inadequate, then the dose will be increased to 8 mg/kg every three weeks for the remaining 12 patients.

Inclusion Criteria

- 1) Females ≤18 years of age
- 2) Histologically confirmed ErbB2 over-expressing metastatic breast cancer
- 3) Patients who have been newly diagnosed with metastatic disease
- 4) Have a Karnofsky performance status of ≤70%
- 5) Give written informed consent prior to any study specific screening procedures with the understanding that the patient has the right to withdraw from the study at any time, without prejudice.

Exclusion Criteria

- 1) Pregnant or lactating women
- 2) Women of childbearing potential unless (1) surgically sterile or (2) using adequate measures of contraception such as oral contraceptive, intra-uterine device or barrier method of contraception in conjunction with spermicidal jelly.
- 3) Clinical or radiologic evidence of CNS metastases.
- 4) History of any significant cardiac disease
- 5) LVEF ≤50%

48

- 6) No prior taxane therapy in any treatment setting.
 - 7) Any of the following abnormal baseline hematologic values:
 - Hb less than 9 g/dl
 - WBC less than $3.0 \times 10^9/l$
 - Granulocytes less than $1.5 \times 10^9/l$
 - Platelets less than $100 \times 10^9/l$
 - 8) Any of the following abnormal baseline liver function tests:
 - Serum bilirubin greater than $1.5 \times ULN$ (upper normal limit)
 - ALT and/or AST greater than $2.5 \times ULN$ (greater than $4.0 \times ULN$ if liver or bone metastasis)
 - Alkaline phosphatase greater than $2.5 \times ULN$ (greater than $4.0 \times ULN$ if liver or bone metastasis)
 - 9) The following abnormal baseline renal function tests:
 - serum creatinine greater than $1.5 \times ULN$
 - 10) History of other serious medical conditions that would preclude patient participation in an investigational study.
- HERCEPTIN®
- Loading dose and schedule: 8 mg/kg for first dose.
Maintenance dose and schedule: 6 mg/kg every 3 weeks.
Paclitaxel—
175 mg/m² IV every 3 weeks×6 cycles as a 3-hour infusion.
- NOTE: On the first cycle of treatment, paclitaxel will be dosed 8 hours prior to HERCEPTIN® to determine the PK of paclitaxel alone. HERCEPTIN® will be administered 8 hours post-paclitaxel for the 1st cycle only. In subsequent treatment cycles, HERCEPTIN® will be administered prior to paclitaxel.
- The total duration of this study is 18 weeks. Study subjects will receive up to 6 total HERCEPTIN® doses. After the last subject has received the last cycle of paclitaxel, data collection for safety and pharmacokinetic analysis will stop, and the study will close to protocol specified treatment. Study subjects may continue to receive the HERCEPTIN®+/-paclitaxel at the discretion of the investigator.
- It is believed that the above treatment regimen will be effective in treating metastatic breast cancer, despite the infrequency with which HERCEPTIN® is administered to the patient.
- While the particular aspects and embodiments of the invention as herein shown and disclosed in detail is fully capable of obtaining the objects and providing the advantages herein before stated, it is to be understood that it is merely illustrative of some of the presently preferred embodiments of the invention and that no limitations are intended to the details of methods and articles of manufacture shown other than as described in the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

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US 10,160,811 B2

51

52

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65

US 10,160,811 B2

53

54

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 Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Val Gln Ala
 65 70 75 80
 Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr
 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
 100 105

<210> SEQ ID NO 11
 <211> LENGTH: 119
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Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Thr
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 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Thr Phe Thr Asp Tyr
 20 25 30
 Thr Met Asp Trp Val Lys Gln Ser His Gly Lys Ser Leu Glu Trp Ile
 35 40 45
 Gly Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
 50 55 60
 Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser Ser Arg Ile Val Tyr
 65 70 75 80
 Met Glu Leu Arg Ser Leu Thr Phe Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr Trp Gly Gln Gly
 100 105 110
 Thr Thr Leu Thr Val Ser Ser
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ile Gly
 20 25 30
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45

US 10,160,811 B2

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Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr
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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr Asp Tyr
20 25 30

Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
50 55 60

Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr Trp Gly Gln Gly
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Thr Leu Val Thr Val Ser Ser
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1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Asn Tyr
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Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
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Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Leu Pro Trp
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Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
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US 10,160,811 B2

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
      20          25          30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
      35          40          45

Ala Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val
      50          55          60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
      65          70          75          80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
      85          90          95

Ala Arg Gly Arg Val Gly Tyr Ser Leu Tyr Asp Tyr Trp Gly Gln Gly
      100          105          110

Thr Leu Val Thr Val Ser Ser
      115

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The invention claimed is:

1. A method for the treatment of a human patient diagnosed with breast cancer characterized by 2+ or 3+ overexpression of ErbB2 receptor as determined by immunohistochemistry or fluorescence in situ hybridization (FISH), comprising the steps of administering to the patient an initial dose of 8 mg/kg of anti-ErbB2 huMAb 4D5-8 antibody; and administering to the patient a plurality of subsequent doses of 6 mg/kg of the antibody, wherein all doses are separated in time from each other by three weeks.

2. The method of claim 1, further comprising administering an effective amount of a chemotherapeutic agent.

3. The method of claim 2, wherein said chemotherapeutic agent is a taxoid.

4. The method of claim 3, wherein said taxoid is paclitaxel or docetaxel.

5. The method of claim 4 wherein said taxoid is paclitaxel.

6. The method of claim 1, wherein said antibody is administered by intravenous injection.

7. A method for the treatment of a human patient diagnosed with breast cancer characterized by 2+ or 3+ overexpression of ErbB2 receptor as determined by immunohistochemistry or fluorescence in situ hybridization (FISH), the

method comprising: administering intravenously to the patient an initial dose of 8 mg/kg of anti-ErbB2 huMAb 4D5-8 antibody; and administering intravenously to the patient a plurality of subsequent 6 mg/kg doses of the antibody, wherein the initial dose is separated in time from the first subsequent dose by three weeks, and the subsequent doses are separated from each other in time by three weeks.

8. The method of claim 7, wherein the intravenous administration is an intravenous infusion.

9. The method of claim 8, wherein the subsequent doses maintain a trough serum concentration of the anti-ErbB2 huMAb 4D5-8 antibody at or above 10 µg/mL.

10. The method of claim 8, wherein the subsequent doses maintain a trough serum concentration of the anti-ErbB2 huMAb 4D5-8 antibody at or above 20 µg/mL.

11. The method of claim 7, wherein the subsequent doses maintain a trough serum concentration of the anti-ErbB2 huMAb 4D5-8 antibody at or above 10 µg/mL.

12. The method of claim 7, wherein the subsequent doses maintain a trough serum concentration of the anti-ErbB2 huMAb 4D5-8 antibody at or above 20 µg/mL.

* * * * *

EXHIBIT F



US007993834B2

(12) **United States Patent
Mass**(10) **Patent No.: US 7,993,834 B2**
(45) **Date of Patent: Aug. 9, 2011**(54) **DETECTION OF ERBB2 GENE
AMPLIFICATION TO INCREASE THE
LIKELIHOOD OF THE EFFECTIVENESS OF
ERBB2 ANTIBODY BREAST CANCER
THERAPY**(75) Inventor: **Robert D. Mass**, Mill Valley, CA (US)(73) Assignee: **Genentech, Inc.**, South San Francisco,
CA (US)(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 276 days.(21) Appl. No.: **11/942,449**(22) Filed: **Nov. 19, 2007**(65) **Prior Publication Data**

US 2008/0112958 A1 May 15, 2008

Related U.S. Application Data(60) Continuation of application No. 11/441,995, filed on
May 26, 2006, now abandoned, which is a division of
application No. 09/863,101, filed on May 18, 2001,
now abandoned.(60) Provisional application No. 60/205,754, filed on May
19, 2000.(51) **Int. Cl.**
C12Q 1/68 (2006.01)
G01N 33/574 (2006.01)(52) **U.S. Cl.** **435/6; 435/7.23**(58) **Field of Classification Search** None
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**

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Primary Examiner — Laura B Goddard(74) *Attorney, Agent, or Firm* — Arnold & Porter LLP;
Diane Marschang; Ginger R. Dreger(57) **ABSTRACT**

The invention provides a method for more effective treatment of patients susceptible to or diagnosed with tumors overexpressing ErbB, as determined by a gene amplification assay, with an ErbB antagonist. Such method comprises administering a cancer-treating dose of the ErbB antagonist, preferably in addition to chemotherapeutic agents, to a subject in whose tumor cells ErbB has been found to be amplified e.g., by fluorescent in situ hybridization. ErbB antagonists described include an anti-HER2 antibody. Pharmaceutical packaging for providing the components for such treatment is also provided.

6 Claims, No Drawings

US 7,993,834 B2

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US 7,993,834 B2

1

**DETECTION OF ERBB2 GENE
AMPLIFICATION TO INCREASE THE
LIKELIHOOD OF THE EFFECTIVENESS OF
ERBB2 ANTIBODY BREAST CANCER
THERAPY**

This is a continuation of application Ser. No. 11/441,995 filed on May 26, 2006 (now abandoned), which is a divisional application of non-provisional application Ser. No. 09/863,101 filed on May 18, 2001 (now abandoned), which claims priority under 35U.S.C. §119(e) to provisional application Ser. No. 60/205,754, filed on May 19, 2000, the entire disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention concerns the treatment of cancers characterized by the overexpression of a tumor antigen, such as an ErbB receptor, particularly HER2. More specifically, the invention concerns more effective treatment of human patients susceptible to or diagnosed with cancer, in which the tumor cells overexpress ErbB as determined by a gene amplification assay, with an ErbB antagonist, e.g., an anti-ErbB antibody. The invention further provides pharmaceutical packages for such treatment.

BACKGROUND OF THE INVENTION

Advancements in the understanding of genetics and developments in technology and epidemiology have allowed for the correlation of genetic abnormalities with certain malignancies as well as risk assessment of an individual for developing certain malignancies. However, most of the methodologies available for evaluation of tissue for the presence of genes associated with or predisposing an individual to a malignancy have well-known drawbacks. For example, methods that require disaggregation of the tissue, such as Southern, Northern, or Western blot analysis, are rendered less accurate by dilution of the malignant cells by the normal or otherwise non-malignant cells that are present in the same tissue. Furthermore, the resulting loss of tissue architecture precludes the ability to correlate malignant cells with the presence of genetic abnormalities in a context that allows morphological specificity. This issue is particularly problematic in tissue types known to be heterogeneous, such as in human breast carcinoma, where a significant percentage of the cells present in any area may be non-malignant.

The her2/neu gene encodes a protein product, often identified as p185HER2. The native p185HER2 protein is a membrane receptor-like molecule with homology to the epidermal growth factor receptor (EGFR). Amplification and overexpression of HER2 in human breast cancer has been correlated with shorter disease-free interval and shorter overall survival in some studies (van de Vijver et al. *New Eng. J. Med.* 319:1239 (1988); Walker et al. *Br. J. Cancer* 60:426 (1989); Tandon et al. *J. Clin. Invest.* 7:1120 (1989); Wright et al. *Cancer Res.* 49:2087 (1989); McCann et al. *Cancer Res.* 51:3296 (1991); Paterson et al. *Cancer Res.* 51:556 (1991); and Winstanley et al. *Br. J. Cancer* 63:447 (1991)) but not in others (Zhou et al. *Oncogene* 4:105 (1989); Heintz et al. *Arch Path Lab Med* 114:160 (1990); Kury et al. *Eur. J. Cancer* 26:946 (1990); Clark et al. *Cancer Res.* 51:944 (1991); and Ravdin et al. *J. Clin. Oncol.* 12:467-74 (1994)).

In an initial evaluation of 103 patients with breast cancer, those having more than three tumor cell positive axillary lymph nodes (node positive) were more likely to overexpress HER2 protein than patients with less than three positive nodes

2

(Slamon et al. *Science* 235:177 (1987)). In a subsequent evaluation of 86 node-positive patients with breast cancer, there was a significant correlation among the extent of gene amplification, early relapse, and short survival. HER2 overexpression was determined using Southern and Northern blotting, which correlate with the HER2 oncoprotein expression evaluated by Western blotting and immunohistochemistry (IHC) (Slamon et al. *Science* 235:177 (1987); Slamon et al. *Science* 244:707 (1989)). The median period of survival was found to be approximately 5-fold shorter in patients with more than five copies of the her2 gene than in patients without gene amplification. This correlation was present even after correcting for nodal status and other prognostic factors in multivariate analyses. These studies were extended in 187 node-positive patients and indicated that gene amplification, increased amounts of mRNA (determined by Northern blotting), and increased protein expression (determined immunohistochemically) were also correlated with shortened survival time (Slamon et al. *Science* 244:707 (1989)); (see also U.S. Pat. No. 4,968,603). Nelson et al. have compared her2/neu gene amplification using FISH with immunohistochemically determined overexpression in breast cancer (Nelson et al. *Modern Pathology* 9 (1) 21A (1996)).

Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing alteration of proteins in a heterogeneous tissue. Immunohistochemistry (IHC) techniques utilize an antibody to probe and visualize cellular antigens in situ, generally by chromagenic or fluorescent methods. This technique excels because it avoids the unwanted effects of disaggregation and allows for evaluation of individual cells in the context of morphology. In addition, the target protein is not altered by the freezing process.

However, in the clinical trial assay (CTA), IHC of formaldehyde-fixed, paraffin embedded tissue samples only demonstrated 50%-80% sensitivity, relative to frozen IHC samples (Press, *Cancer Research* 54:2771 (1994)). Thus, IHC can lead to false negative results, excluding from treatment patients who might benefit from the treatment.

Fluorescence in situ hybridization (FISH) is a recently developed method for directly assessing the presence of genes in intact cells. FISH is an attractive means of evaluating paraffin-embedded tissue for the presence of malignancy because it provides for cell specificity, yet overcomes the cross-linking problems and other protein-altering effects caused by formalin fixation. FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology (see, e.g., Anastasi et al., *Blood* 77:2456-2462 (1991); Anastasi et al., *Blood* 79:1796-1801 (1992); Anastasi et al., *Blood* 81:1580-1585 (1993); van Lom et al., *Blood* 82:884-888 (1992); Wolman et al., *Diagnostic Molecular Pathology* 1(3):192-199 (1992); Zitzelberger, *Journal of Pathology* 172:325-335 (1994)).

To date, there has been no correlation of her2 gene amplification with anti-HER2 antibody treatment outcome, only with disease prognosis. The standard assay has been IHC on formalin fixed, paraffin embedded samples. These samples, when scored as 3+ or 2+, identify patients who are likely to benefit from treatment with an anti-HER2 antibody, like Herceptin®. The 3+ and 2+ scores correlate with her2 gene amplification, e.g., as tested by FISH. However, there remains a need for more effective identification of candidates for successful ErbB antagonist therapies, such as Herceptin® treatment. SUMMARY OF THE INVENTION

The invention advantageously provides a method for increasing likelihood of effectiveness of an ErbB antagonist cancer treatment. The method comprises administering a can-

US 7,993,834 B2

3

cer treating dose of the ErbB antagonist to a subject wherein an erbB gene in tumor cells in a tissue sample from the subject has been found to be amplified. Preferably the ErbB is HER2. In a specific embodiment, the method further comprises administering a cancer treating dose of a chemotherapeutic, particularly a taxol.

In a specific preferred embodiment, exemplified herein, the invention provides a method for increasing likelihood of effectiveness of an anti-HER2 antibody to treat cancer. This method comprises administering a cancer treating dose of the anti-HER2 antibody to the subject in whom a her2 gene in tumor cells in a tissue sample from the subject have been found to be amplified.

The unexpected clinical results underlying the invention, in which gene amplification proved to be a more effective indication of antibody-based tumor therapy than protein detection by immunohistochemistry, extends to tumor antigens in general. Thus, any anti-tumor-specific antigen based antibody therapy can have increased likelihood of success in patients who are found to have gene amplification of the gene encoding the tumor antigen.

A particular advantage of the invention is that it permits selection of patients for treatment who, based on immunohistochemical criteria, would be excluded. Thus, in a specific embodiment, the subject has been found to have an antigen level corresponding to a 0 or 1+ score for HER2 by immunohistochemistry on a formaldehyde-fixed tissue sample.

The invention further provides a pharmaceutical package comprising an ErbB antagonist for treating a cancer, and instructions to administer the ErbB antagonist to a subject if an erbB gene in tumor cells in a tissue sample from the subject is amplified. Preferably the ErbB antagonist is an anti-ErbB antibody, such as an anti-HER2 antibody. In a further aspect, the instructions also teach administering a cancer treating dose of a chemotherapeutic, e.g., a taxol. Such pharmaceutical packages, including the instructions for use, can be provided for any antibody-based therapeutic specific for a tumor-specific antigen.

DETAILED DESCRIPTION

The present invention advantageously permits treatment of patients who have a greater likelihood of responding to the treatment by administering therapeutic agents, i. e., anti-tumor antigen therapeutic antibodies or ErbB receptor antagonists, to patients who are found to have an amplified gene encoding such a tumor antigen or ErbB receptor protein. The invention is based, in part, on the unexpected discovery that her2 gene amplification, e.g., as detected by fluorescence in situ hybridization (FISH), although it correlates with HER2 expression as detected by immunohistochemistry (IHC), provides a more accurate basis for selecting patients for treatment because FISH status unexpectedly correlates better with response to treatment. This outcome was surprising in part because FISH status has about the same rate of correlation with a clinical trial assay (CTA) IHC assay as another IHC assay (HercepTest). Based on this observation, FISH would be expected to have a similar correlation with treatment response. This outcome also surprises because direct measurement of protein (by immunoassay) would be expected to provide a more accurate assessment of a cancer therapy targeted to the protein than an indirect measure of expression, like gene amplification.

Evaluation of patient groups and subgroups demonstrates the power of gene amplification analysis for selecting patients more likely to respond to treatment. IHC provides a score for HER2 expression on tumor cells: 0 (no expression) through

4

3+ (very high levels of expression). Clinical selection criteria exclude patients with 0 and 1+ scores and select patients with 2+ and 3+ scores. The data show that 14% of combined 2+/3+ patients respond to Herceptin®, while 20% of FISH+ (amplified her2 gene) patients respond to Herceptin®. The 3+ subgroup has a 17% response rate, which is very close to the FISH+ subjects' response rate. However, the 2+ subgroup has less than half the response rate of FISH+ subjects. Thus, gene amplification clearly differentiates large sub-populations within the 2+ subgroup, permitting more effective treatment for those who are FISH+, and quickly identifying patients for whom alternative treatment modalities are appropriate and should commence immediately.

Gene amplification analysis also identifies patients who are unnecessarily excluded because of anomalies in the IHC analysis, particularly when the tests are performed on formalin fixed, paraffin embedded samples (such sample processing can disrupt or destroy antibody epitopes on the HER2 protein, but has much less impact on gene amplification assays). As shown in the examples, a subset of 0 and 1+ subjects are FISH+. These patients are likely to respond to anti-HER2 antibody therapy, e.g., with Herceptin®, although by IHC criteria they would be excluded from receiving this treatment.

Thus, the present invention advantageously permits inclusion of patients who are more likely to benefit from treatment but who, by standard IHC criteria, would be excluded from treatment. At the same time, the invention permits exclusion of patients who should promptly seek an alternative mode of treatment because the anti-tumor antigen therapy (i.e., ErbB antagonist or tumor antigen-specific therapeutic antibody) is not likely to succeed.

In short, the present invention is a powerful adjunct to IHC assays for target protein expression level-based selection of patients. It can also be employed on its own, i.e., without IHC, to provide initial screening and selection of patients. The invention significantly improves screening and selection for subjects to receive a cancer-treating dose of an anti-tumor antigen therapeutic antibody treatment, ErbB receptor antagonist treatment, and other treatment targeted to overexpressed tumor antigens (or tumor-specific antigens), resulting in an increased likelihood of benefit from such treatments.

In another aspect, the invention concerns an article of manufacture or package, comprising a container, a composition within the container comprising an ErbB antagonist, e.g., an anti-ErbB antibody (or other anti-tumor-specific antigen antibody), optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB receptor, and a package insert containing instructions to administer the antagonist to patients who have been found to have an amplified erbB gene.

Definitions

As used herein, an "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3, and ErbB4 receptors, as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid

US 7,993,834 B2

5

sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

ErbB receptors are examples of tumor-specific antigens or tumor antigens. The term "tumor antigen" is used herein to refer to a protein that is expressed at a higher level on tumor cells compared to normal cells. Generally, the normal cells for comparison are of the same tissue type, particularly phenotype, as the tumor, or from which the tumor arose. A "tumor specific antigen" refers to an antigen expressed either preferentially or only on tumor cells. Examples of tumor-specific antigens include, in addition to the ErbB receptors, MART1/Melan A, gp-100, and tyrosinase (in melanoma); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin/MUC-1 (in breast, pancreas, colon, and prostate cancers); prostate specific antigen/PSA (in prostate cancer); and carcinoembryonic antigen/CEA (in colon, breast, and gastrointestinal cancers).

By "amplification" is meant the presence of one or more extra gene copies of erbB or other tumor antigen-encoding gene in a chromosome complement. Gene amplification can result in overexpression of protein, e.g., ErbB receptor protein. Gene amplification in cells from a tissue sample can be measured by many techniques, particularly Fluorescence in situ Hybridization (FISH), but also including and not limited to quantitative PCR, quantitative Southern hybridization, and the like.

By "tissue sample" is meant a collection of similar cells obtained from a tissue of a subject or patient, preferably containing nucleated cells with chromosomal material. The four main human tissues are (1) epithelium; (2) the connective tissues, including blood vessels, bone and cartilage; (3) muscle tissue; and (4) nerve tissue. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like. In one embodiment of the invention, the tissue sample is "non-hematologic tissue" (i.e., not blood or bone marrow tissue).

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample may be analyzed at both morphological and molecular levels, or may be analyzed with respect to both protein and nucleic acid.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis with the performance and/or results of a second analysis. For example, one may use the results of a first analysis in carrying out the second analysis and/or one may use the results of a first analysis to determine whether a second analysis should be performed and/or one may compare the results of a first analysis with the results of a second analysis. In relation to IHC combined with FISH, one may use the results of IHC to determine whether FISH should be performed and/or one may compare the level of protein expression with gene amplification to further characterize a tumor biopsy (e.g. to compare HER2 protein expression with her2 gene amplification).

6

One advantageous feature of the invention is the ability to identify patients likely to benefit from treatment using FISH even if IHC indicates that they are antigen low.

By "nucleic acid" is meant to include any DNA or RNA, for example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing an RNA (rRNA, tRNA, or mRNA, the latter capable of translation as a protein) or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); Transforming Growth Factor alpha (TGF-alpha) (Marquardt et al., Science 223:1079-1082 (1984)); amphiregulin, also known as schwannoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al., Science 259:1604-1607 (1993); and Sasada et al. Biochem. Biophys. Res. Commun. 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939 (1991)); epiregulin (Toyoda et al., J. Biol. Chem. 270:7495-7500 (1995); and Komurasaki et al. Oncogene 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., Proc. Natl. Acad. Sci. 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al., J. Biol. Chem. 272(6):3330-3335 (1997)). ErbB ligands that bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide comprising an amino acid sequence encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869 or Marchionni et al., Nature, 362:312-318 (1993), and biologically active variants of such polypeptides. Examples of heregulins include heregulin-alpha, heregulin-beta1, heregulin-beta2, and heregulin-beta3 (Holmes et al., Science, 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69:205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Cell 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., Nature, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. J. Biol. Chem. 270:14523-14532 (1995)); gamma-heregulin (Schaefer et al. Oncogene 15:1385-1394 (1997)). An example of a biologically active fragment/amino acid sequence variant of a native sequence HRG polypeptide, is an EGF-like domain fragment (e.g., HRG-beta1, 177-244).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be

US 7,993,834 B2

7

isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., (J. Biol. Chem., 269(20):14661-14665 (1994)), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3, and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4, or EGFR. Other proteins, such as a cytokine receptor subunit (e.g., gp130), may be included in the hetero-oligomer.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. (Ann. Rev. Biochem. 56:881-914 (1987)), including variants thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (Proc. Natl. Acad. Sci. U.S.A. 87:4207-4211 (1990)). ErbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), Mab 455 (ATCC CRL HB 8507), Mab 225 (ATCC CRL 8508), Mab 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, PCT Publication No. WO 96/40210).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., (Proc. Natl. Acad. Sci. U.S.A. 82:6497-6501 (1985)) and Yamamoto et al. (Nature 319:230-234(1986)) (Genebank accession number X03363), and variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat p185neu. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include Mabs 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB-12215) (see, U.S. Pat. No. 5,772,997; PCT Publication No. WO 98/17797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, and huMAb4D5-8 (Herceptin®) as described in Table 3 of U.S. Pat. No. 5,821,337, which is expressly incorporated herein by reference; and humanized 520C9 (PCT Publication No. WO 93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 and PCT Publication No. WO 97/00271.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968, as well as Kraus et al. (Proc. Natl. Acad. Sci. U.S.A.) 86:9193-9197 (1989)), including variants thereof. Exemplary antibodies that bind HER3 are described in U.S. Pat. No. 5,968,511, e.g., the 8B8 antibody (ATCC HB-12070) or a humanized variant thereof. The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in European Application No. EP 599,274; Plowman et al., (Proc. Natl. Acad. Sci. U.S.A., 90:1746-1750 (1993)); and Plowman et al., (Nature, 366:473-475 (1993)), including variants thereof such as the HER4 isoforms disclosed in PCT Publication No. WO 99/19488.

An "ErbB antagonist" is any molecule that binds to an ErbB receptor and blocks ligand activation of the ErbB receptor. Such antagonists include, but are not limited to, modified ligands, ligand peptides (i.e., ligand fragments), soluble ErbB receptors, and, preferably, anti-ErbB antibodies.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

8

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the ErbB antagonist, e.g., anti-ErbB2 antibody, and more generally, any cancer in which administration of an antibody against an over-expressed antigen can treat the cancer. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount elicits antibody-mediated cytotoxicity, activates complement, has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time for disease progression (TTP), survival, tumor size, or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, melanoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small-cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface, such that an anti-ErbB antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I¹³¹, I¹²⁵, Y⁹⁰, and Re¹⁸⁶), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as ben-zodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibi-

US 7,993,834 B2

9

otics such as aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, pectinomycin, pottiomycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitioestanol, mepitioestane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglutone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguanzone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procabazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepe; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; carminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and anti-androgens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), espe-

10

cially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

ErbB Receptor Tyrosine Kinases

The ErbB receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes at least four distinct members including Epidermal Growth Factor Receptor (EGFR or ErbB1), HER2 (ErbB2 or p185neu), HER3 (ErbB3), and HER4 (ErbB4 or tyro2).

EGFR, encoded by the ErbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer, as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, Transforming Growth Factor alpha (TGF-alpha), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-alpha and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn, *supra*; Masui et al. *Cancer Research* 44:1002-1007 (1984); and Wu et al. *J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the ErbB family, p185neu, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science*, 235:177-182(1987); Slamon et al., *Science*, 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder.

Antibodies directed against the rat p185neu and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185neu (see, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and W094/22478). Drebin et al. (*Oncogene* 2:273-277(1988)) report that mixtures of antibodies reactive with two distinct regions of p185neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice (see also U.S. Pat. No. 5,824,311).

Hudziak et al., (*Mol. Cell. Biol.* 9(3):1165-1172 (1989)) describe the generation of a panel of anti-HER2 antibodies, which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5, which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-alpha (see, also, U.S. Pat. No. 5,677,171). The anti-HER2 antibodies discussed in Hudziak et al. were further characterized (Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):

US 7,993,834 B2

11

59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997)).

A recombinant humanized IgG1 version of the murine anti-HER2 antibody 4D5 (rhuMAb HER2 or Herceptin®; commercially available from Genentech, Inc., South San Francisco) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Herceptin® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. The current treatment protocol employs IHC to determine HER2 protein overexpression.

Other anti-HER2 antibodies with various properties have been described (Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., (*Proc. Natl. Acad. Sci. U.S.A.*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); PCT Publication No. WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; Klapper et al. *Oncogene* 14:2099-2109 (1997); and PCT Publication No. WO 98/17797).

Homology screening has resulted in the ErbB receptor family members: HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968; Kraus et al., *Proc. Natl. Acad. Sci. U.S.A.* 86:9193-9197 (1989)) and HER4 (European Patent Application No. EP 599 274; Plowman et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:1746-1750 (1993); and Plowman et al., *Nature*, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al., *Breast Cancer Research and Treatment* 35:115-132 (1995)). EGFR is bound by six different ligands: Epidermal Growth Factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, Heparin Binding Epidermal Growth Factor (HB-EGF), betacellulin, and epieregulin (Groenen et al. *Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta, and gamma heregulins (Holmes et al., *Science*, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al., *Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motoneuron derived factor (SMDF) (for a review, see Groenen et al., *Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee et al. *Pharm. Rev.* 47:51-85 (1995)). Recently, two additional ErbB ligands were identified: neuregulin-2

12

(NRG-2), which is reported to bind either HER3 or HER4 (Chang et al., *Nature*: 387 509-512 (1997); and Carraway et al. *Nature* 387:512-516 (1997)) and neuregulin-3, which binds HER4 (Zhang et al., (*Proc. Natl. Acad. Sci. U.S.A.*) 94(18): 9562-7 (1997)). HB-EGF, betacellulin, and epieregulin also bind to HER4.

While EGF and TGF-alpha do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase, (Earp et al., supra.) Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., *Journal of Neuroscience* 15:1329-1340 (1995); Morrissey et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:1431-1435 (1995); and Lewis et al., *Cancer Res.*, 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8(1994)).

Detecting Gene Amplification

The present invention contemplates using any technique to detect gene amplification. (see, Boxer, *J. Clin. Pathol.* 53:19-21(2000)). These techniques include in situ hybridization (Stoler, *Clin. Lab. Med.* 12:215-36 (1990), using radioisotope or fluorophore-labeled probes; polymerase chain reaction (PCR); quantitative Southern blotting, and other techniques for quantitating individual genes. Preferably probes or primers selected for gene amplification evaluation are highly specific, to avoid detecting closely related homologous genes.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. A hapten or epitope that is immunospecifically bound by an antibody can also serve as a label.

The term "fluorescently labeled nucleic acid probe" refers to a probe comprising (1) a nucleic acid having a sequence rendering it capable of hybridizing with a target nucleic acid sequence and (2) a fluorescent label. Preferably such hybridization is specific, i. e., it can occur under high stringency conditions.

Sample Preparation

Any tissue sample from a subject may be used. Examples of tissue samples that may be used include, but are not limited to, breast, prostate, ovary, colon, lung, endometrium, stomach, salivary gland or pancreas. The tissue sample can be obtained by a variety of procedures including, but not limited to surgical excision, aspiration, or biopsy. The tissue may be fresh or frozen. In one embodiment, the tissue sample is fixed and embedded in paraffin or the like.

The tissue sample may be fixed (i.e., preserved) by conventional methodology (See e.g., *Manual of Histological Staining Method of the Armed Forces Institute of Pathology*, 3rd Edition Lee G. Luna, HT (ASCP) Editor, The Blakston Division McGraw-Hill Book Company: New York; (1960);

US 7,993,834 B2

13

The Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology (1994) Ulreka V. Mikel, Editor, Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.). One of skill in the art will appreciate that the choice of a fixative is determined by the purpose for which the tissue is to be histologically stained or otherwise analyzed. One of skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin's or paraformaldehyde, may be used to fix a tissue sample.

Generally, the tissue sample is first fixed and is then dehydrated through an ascending series of alcohols, infiltrated, and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. By way of example, the tissue sample may be embedded and processed in paraffin by conventional methodology. Examples of paraffin that may be used include, but are not limited to, Paraplast, Broloid, and Tissuemay. Once the tissue sample is embedded, the sample may be sectioned by a microtome or the like. By way of example for this procedure, sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine, and the like. For example, the paraffin embedded sections may be attached to positively charged slides, slides coated with poly-L-lysine.

If paraffin has been used as the embedding material, the tissue sections are generally deparaffinized and rehydrated to water. The tissue sections may be deparaffinized by several conventional standard methodologies. For example, xylenes and a gradually descending series of alcohols may be used. Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Tex.) may be used.

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is generally carried out on cells or tissue sections fixed to slides. In situ hybridization may be performed by several conventional methodologies (see, e.g., Leitch et al., *In Situ Hybridization: A Practical Guide*, Oxford BIOS Scientific Publishers, Microscopy Handbooks v. 27 (1994)). In one in situ procedure, fluorescent dyes (such as fluorescein isothiocyanate (FITC) which fluoresces green when excited by an Argon ion laser) are used to label a nucleic acid sequence probe that is complementary to a target nucleotide sequence in the cell. Each cell containing the target nucleotide sequence will bind the labeled probe producing a fluorescent signal upon exposure, of the cells to a light source of a wavelength appropriate for excitation of the specific fluorochrome used. A "target nucleotide sequence" is a sequence specific for a over-expressed tumor antigen, such as ErbB. FISH analysis can be used in conjunction with other assays, including without limitation morphological staining (of serial sections or the same section; see PCT Publication No. WO 00/20641, specifically incorporated herein by reference).

Various degrees of hybridization stringency can be employed. As the hybridization conditions become more stringent, a greater degree of complementarity is required between the probe and target to form and maintain a stable duplex. Stringency is increased by raising temperature, lowering salt concentration, or raising formamide concentration. Adding dextran sulfate or raising its concentration may also increase the effective concentration of labeled probe to

14

increase the rate of hybridization and ultimate signal intensity. After hybridization, slides are washed in a solution generally containing reagents similar to those found in the hybridization solution with washing time varying from minutes to hours depending on required stringency. Longer or more stringent washes typically lower nonspecific background but run the risk of decreasing overall sensitivity.

Probes used in the FISH analysis may be either RNA or DNA oligonucleotides or polynucleotides and may contain not only naturally occurring nucleotides but their analogs like digoxigenin dCTP, biotin dCTP 7-azaguanosine, azidothymidine, inosine, or uridine. Other useful probes include peptide probes and analogues thereof, branched gene DNA, peptidomimetics, peptide nucleic acid (PNA), and/or antibodies.

Probes should have sufficient complementarity to the target nucleic acid sequence of interest so that stable and specific binding occurs between the target nucleic acid sequence and the probe. The degree of homology required for stable hybridization varies with the stringency of the hybridization medium and/or wash medium. Preferably, completely homologous probes are employed in the present invention, but persons of skill in the art will readily appreciate that probes exhibiting lesser but sufficient homology can be used in the present invention (see e.g., Sambrook, J., et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Press, (1989)).

One of skill in the art will appreciate that the choice of probe depends on the characteristics of the target gene of interest. Examples of amplification include, but are not limited to, her2/neu in breast and ovarian cancer, n-myc in neuroblastoma, c-myc in small cell lung cancer. By way of example for evaluating her2/neu amplification a probe spanning a 140 kb region on the long arm of chromosome 17 containing the her2/neu gene (17q11.2-17q12) may be used. A probe for the -satellite sequences in the centromeric region of chromosome 17(D17Z1) may be used to evaluate for aneuploidy of chromosome 17 as a source or cause for her2/neu amplification. For example, a cocktail version of these probes may be obtained from Vysis, Inc. where each probe is directly labeled with easily distinguishable fluorophores, such as SPECTRUM ORANGE® and SPECTRUM GREEN®.

Probes may also be generated and chosen by several means including, but not limited to, mapping by in situ hybridization, somatic cell hybrid panels, or spot blots of sorted chromosomes; chromosomal linkage analysis; or cloned and isolated from sorted chromosome libraries from human cell lines or somatic cell hybrids with human chromosomes, radiation somatic cell hybrids, microdissection of a chromosome region, or from yeast artificial chromosomes (YACs) identified by PCR primers specific for a unique chromosome locus or other suitable means like an adjacent YAC clone. Probes may be genomic DNA, cDNA, or RNA cloned in a plasmid, phage, cosmid, YAC, Bacterial Artificial Chromosomes (BACs), viral vector, or any other suitable vector. Probes may be cloned or synthesized chemically by conventional methods. When cloned, the isolated probe nucleic acid fragments are typically inserted into a vector, such as lambda phage, pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host (see, e.g., Sambrook, supra).

Probes are preferably labeled with a fluorophore. Examples of fluorophores include, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocyanin, phycocyanin, or commercially available fluorophores such as SPECTRUM ORANGE® and SPECTRUM GREEN®, and/or

US 7,993,834 B2

15

derivatives of any one or more of the above. Multiple probes used in the assay may be labeled with more than one distinguishable fluorescent or pigment color. These color differences provide a means to identify the hybridization positions of specific probes. Moreover, probes that are not separated spatially can be identified by a different color light or pigment resulting from mixing two other colors (e.g., light red+green=yellow), pigment (e.g., blue+yellow=green), or by using a filter set that passes only one color at a time.

Probes can be labeled directly or indirectly with the fluorophor, utilizing conventional methodology. Additional probes and colors may be added to refine and extend this general procedure to include more genetic abnormalities or serve as internal controls. By way of example the her2/neu gene is in chromosome 17, and as an internal control a probe for satellite sequences specific for chromosome 17 (D17Z1) may be used (Vysis, Inc.) to prove diploidy in areas of non-malignant cells and/or to establish the presence or absence of chromosome 17 aneusomy in areas of her2/neu amplification.

After processing for FISH, the slides may be analyzed by standard techniques of fluorescence microscopy (see, e.g., Ploem and Tanke, *Introduction to Fluorescence Microscopy*, Oxford University Press: New York (1987)). Briefly, each slide is observed using a microscope equipped with appropriate excitation filters, dichromic, and barrier filters. Filters are chosen based on the excitation and emission spectra of the fluorochromes used. Photographs of the slides may be taken with the length of time of film exposure depending on the fluorescent label used, the signal intensity and the filter chosen. For FISH analysis the physical loci of the cells of interest determined in the morphological analysis are recalled and visually conformed as being the appropriate area for FISH quantification.

In order to correlate IHC with FISH, one may use a computer-driven, motorized stage which stores location of coordinates. This may be used to evaluate the same area by two different analytical techniques. For example, color images of the morphologically stained areas may be captured and saved using a computer-assisted cooled CCD camera. The same section may be subsequently taken through the FISH procedure, the stored locations recalled, and the designated areas scored for the presence of fluorescent nuclear signals. A similar procedure for IHC followed by FISH is contemplated.

Typically, hundreds of cells are scanned in a tissue sample and quantification of the specific target nucleic acid sequence is determined in the form of fluorescent spots, which are counted relative to the number of cells. Deviation of the number of spots in a cell from a norm (e.g., such as probing for the her2/neu gene in a normal cell will produce two copies, abnormal greater than two) is indicative of a greater likelihood of benefit from a tumor antigen-specific antibody therapy, e.g., an ErbB antagonist therapy. As exemplified infra, her2 gene amplification provides a much more effective indication of the likelihood that an anti-HER2 antibody therapy will be effective.

Pharmaceutical Formulations

Therapeutic formulations of the antagonists, e.g., antibodies, used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buff-

16

ers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICSTM or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, vascular endothelial factor (VEGF), or an antibody that binds to a different epitope on the target ErbB, in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, a chemotherapeutic, a cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.

The formulations to be used for in vivo administration are preferably, and in the case of humans, must be, sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(—)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mecha-

US 7,993,834 B2

17

nism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Treatment with the Anti-ErbB Antagonists

It is contemplated that, according to the present invention, the anti-ErbB antibodies or other antagonists may be used to treat various conditions characterized by overexpression and/or activation of the ErbB receptor in patients who have been found to have an amplified erbB gene. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelec, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and any other active agents of the invention are administered to a human patient in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB antibody and a chemotherapeutic agent, e.g., a taxoid. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells (tumor resection) and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antagonist, e.g. antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by

18

continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Pharmaceutical Packages: Articles of Manufacture

In a related aspect of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, optionally labeled, and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a composition that is effective for treating the condition and preferably has a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-tumor antigen therapeutic antibody or an ErbB antagonist, e.g., an anti-ErbB antibody. A label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. This second buffer can be used to reconstitute the active agent, if that is provided as a lyophilysate or dried powder, or to dilute a concentrated preparation of the active agent. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In addition, the article of manufacture comprises a package insert or inserts with instructions for use in patients who have been found to have erbB gene amplification, e.g., by FISH testing. Such patients may be subjects who, by IHC, would be excluded from treatment with the ErbB antagonist, e.g., patients who score a 0 or 1+ using an anti-HER2 antibody.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., U.S.A. (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLE 1

Concordance Between the Clinical Trials Assay (CTA) and Fluorescence

In Situ Hybridization (FISH) in the Herceptin® Pivotal Trials

Overexpression of HER2 at the 2+ or 3+ level by immunohistochemistry (IHC) was required for enrollment in the

US 7,993,834 B2

19

pivotal Herceptin® metastatic breast cancer trials. The Clinical Testing Assay (CTA) involves two separate IHC assays performed with either monoclonal antibodies 4D5 (after protease digestion of the formalin fixed sample) or CB11 (after heat treatment of the formalin fixed sample). Subjects were eligible if either assay was scored at 2+ or 3+. If both were performed, the final score was the higher of the two results.

Concordance between the CTA and another IHC, Herceptin® test (HT), is 79%. This was the basis for FDA approval of HT to aid in the selection of patients for Herceptin therapy.

This Example describes a similar concordance study, utilizing clinical material submitted for screening for the Herceptin® pivotal trials, that compares the CTA to her2/neu gene amplification measured by the PathVysion FISH assay. In the pivotal trials, 5998 subjects were screened for HER2 expression; 1915 (32%) were positive by the CTA and 4083 (68%) were negative. A random sample of 623 specimens (1:1 ratio of positive:negative) were selected for this analysis, 317 CTA+ and 306 CTA-. Specimens were not freshly cut from blocks. They had been stored between 2 and 4 years as 4-6µ sections on glass slides. Each section was assayed for her2/neu amplification using the protocol specified in the package insert of the PathVysion assay. Amplification was defined as a signal ratio of greater than or equal to 2. The results are shown in Table 1.

TABLE 1

		FISH/CTA Concordance			
		CTA			
		0	1+	2+	3+
FISH	-	207	28	67	21
	+	7	2	21	176
		4%	7%	24%	89%
		529			

FISH+ = HER2:CEP17 signal ratio ≥ 2
Concordance = 82% (79-85%)

For the total 623 specimens tested, a FISH signal result was obtained in 529. Assay failure occurred in 19.9% of CTA- and 10.4% CTA+ samples. Amplification in the 0, 1+, 2, and 3+ groups was 4.2%, 6.7%, 23.9%, and 89.3%, respectively. The sample concordance was 81.3%, similar to the CTA/HT concordance of 79%. Single copy overexpression was 31%, predominantly in the 2+ group. Amplification was rarely (4.6%) noted in the CTA- group. The higher assay failure rate in the CTA- group may be due to non-assay related factors such as tissue fixation. These may have also resulted in false negative results for IHC.

These data were closely interpreted to suggest that her2/neu amplification status may have unexpectedly superior predictive value for identifying patients who are more likely to benefit from Herceptin® treatment as compared to Herceptin® test. The observation that only 24% of 2+ patients are FISH+ suggest that this sub-group may have less predictable treatment outcomes when selected by IHC only. Identification of FISH+ patients in the 1+ and 0 sub-groups might identify subjects who, though failing the IHC criteria for Herceptin® treatment, would likely benefit from Herceptin® treatment. A direct analysis of Herceptin® benefit based on FISH score compared to IHC score is presented in Example 2.

EXAMPLE 2

FISH/Clinical Outcome Study

This example links the results from three Herceptin® Trials with FISH status. In this study, 805 subjects were selected at random from all three trials. Of these, 167 lacked slides.

20

Another 78 assays (9.7%) failed. Thus, formalin-fixed cut sections stored between 2.5 and 4.5 years from 540 subjects provided the sample pool for this study. There were no imbalances in demographics or prognostic indicators in these samples. Results are reported for different treatment groups.

Correlation of FISH status with response was evaluated for patients who received Herceptin® as a second or third line therapy. These data are reported for 2+ and 3+ (by CTA) subjects in Table 2.

TABLE 2

FISH/Response with single agent Herceptin®, 2nd or 3rd line Therapy, 2+/3+ Combined		
	FISH+	FISH-
Response	21	0
No response	84	37
response rate	20% (12.5-27.5%)	0% (0.7%)

N = 142

The 20% response rate of FISH+ subjects unexpectedly exceeds the 15% response rate of 2+ and 3+ patients in this study and 14% response rate observed in patients selected by CTA with a 2+ or 3+ immunohistochemistry score during the pivotal trials. Thus, while FISH correlates well with IHC to about the same degree as another IHC assay, the Hercept Test, as shown in Example 1, it unexpectedly is superior in identifying patients who are more likely to benefit from Herceptin® therapy.

When these data were broken down into the components 3+ and 2+ subjects, the same 20% response rate of FISH+ subjects was seen (Tables 3 and 4).

TABLE 3

FISH/Response with single agent Herceptin®, 2nd or 3rd line therapy, 3+ subgroup		
	FISH+	FISH-
Response	18	0
No response	72	17
response rate	20% (12-28%)	0% (0-14%)

N = 107

TABLE 4

FISH/Response with single agent Herceptin®, 2nd or 3rd line therapy, 2+ subgroup		
	FISH+	FISH-
Response	3	0
No response	12	20
response rate	20% (1-40%)	0% (0-14%)

N = 35

In the 3+ sub-group, the FISH+ response rate (20%) was very close but still exceeded the 17% response rate of 3+ subjects. The 2+ subgroup showed a much greater difference, with only a 9% response rate versus 20% by FISH+ selection. These data show that FISH+ status (her2 gene amplification) greatly increases the likelihood of response to Herceptin®.

US 7,993,834 B2

21

Data were also evaluated for patient responses to Herceptin® as a first line therapy (Table 5).

TABLE 5

FISH/Response with single agent Herceptin® as 1st line therapy, 2+/3+ combined		
	FISH+	FISH-
Response	17	1
No response	24	20
response rate	41% (26-56%)	20% (0-14%)

N = 62

The 41% response rate of FISH+ subjects was notably greater than the 27% response rate of 3+, 2+, subjects.

The surprising increase in likelihood of beneficial response based on FISH analysis extended to responses to chemotherapy plus Herceptin®, as shown in Table 6. FISH+ subjects showed a much greater response to chemotherapy and Herceptin® (54%) than FISH-(41%). Tables 7-9 contain more extensive data, broken down by different chemotherapeutic agents (adriamycin and cyclophosphamide, AC; and Paclitaxel, P) and different endpoints (response rate, time to progression, and survival) for Herceptin® in combination with chemotherapy.

TABLE 6

FISH/Response rate to chemotherapy +/- Herceptin®, 1st line therapy, 2+/3+ combined		
	C alone	C + H
FISH-	39% (26-52%)	41% (27-55%)
FISH+	27% (19-35%)	54% (45-63%)

N = 336

TABLE 7

Response rate of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	56*	42	41 *	17	50*	32
3+	349	60*	42	49*	17	56*	31
FISH+	240	58*	40	49*	14	54*	27

*p < 0.05

TABLE 8

Time to progression (months) of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	7.8*	6.1	6.9*	2.7	7.4*	4.6
3+	349	8.1*	6.0	7.1*	3.0	7.8*	4.6
FISH+	240	7.8*	6.2	7.0*	3.2	7.3*	4.6

*p < 0.05

22

TABLE 9

Survival (months) of newly defined populations							
	H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)	
2+/3+	469	27	21	22	18	25*	20
3+	349	31*	21	25	18	29*	20
FISH+	240	29*	20	25*	14	27*	18

*p < 0.05

These data uniformly confirm that FISH+ analysis, though correlating closely to IHC, provides a much more accurate indicator of likelihood of success with Herceptin® treatment. Across the board, FISH+ selection has about 1/3 (30%) greater response rate than 2+/3+ IHC-selection. When focused on 2+ patients, FISH status provides a much more effective tool for patient selection. FISH states also identifies patients who, because of 0 or 1+ status as determined by IHC, would otherwise be excluded from treatment.

These observations have broad implications for ErbB receptor antagonist-based cancer therapies and anti-tumor antigen cancer therapies in general. Thus erbB antagonists, e.g., anti-erbB receptor antibodies like Herceptin®, can have an increased likelihood of efficacy when administered to patients who are positive for erbB gene amplification, e.g., by a FISH test. This is certainly the case, based on these data, with Herceptin®.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed:

1. A method for increasing likelihood of effectiveness of breast cancer treatment with humanized anti-ErbB2 antibody huMAb4D5-8, which method comprises administering a cancer treating dose of said antibody to a human subject diagnosed with breast cancer, wherein an erbB2 gene amplification in breast cancer cells in a tissue sample from the subject has been detected, and wherein the breast cancer cells from the human subject have been found to have a 0 or 1+ score of ErbB2 protein expression by immunohistochemistry.

2. The method according to claim 1, wherein the breast cancer cells from the human subject have been found to have a 0 or 1+ score of ErbB2 protein expression by immunohistochemistry on a formaldehyde-fixed tissue sample.

3. The method according to claim 1 wherein the erbB2 gene amplification is detected by detecting fluorescence of a fluorescent-labeled nucleic acid probe hybridized to the gene.

4. The method according to claim 1, which further comprises administering a cancer treating dose of a chemotherapeutic drug.

5. The method according to claim 4, wherein the chemotherapeutic drug is a taxoid.

6. The method according to claim 1 wherein the likelihood of effectiveness increases by about 30%.

* * * * *

EXHIBIT G



US008076066B2

(12) **United States Patent
Mass**(10) **Patent No.: US 8,076,066 B2**
(45) **Date of Patent: *Dec. 13, 2011**(54) **GENE DETECTION ASSAY FOR IMPROVING
THE LIKELIHOOD OF AN EFFECTIVE
RESPONSE TO A HER2 ANTIBODY CANCER
THERAPY**(75) Inventor: **Robert D. Mass**, Mill Valley, CA (US)(73) Assignee: **Genentech, Inc.**, South San Francisco,
CA (US)(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.This patent is subject to a terminal dis-
claimer.(21) Appl. No.: **11/690,304**(22) Filed: **Mar. 23, 2007**(65) **Prior Publication Data**

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Related U.S. Application Data(63) Continuation of application No. 09/863,101, filed on
May 18, 2001, now abandoned.(60) Provisional application No. 60/205,754, filed on May
19, 2000.(51) **Int. Cl.**
C12Q 1/68 (2006.01)
G01N 33/574 (2006.01)(52) **U.S. Cl.** **435/6; 435/7.23**(58) **Field of Classification Search** None
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**

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Primary Examiner — Laura B Goddard(74) *Attorney, Agent, or Firm* — Diane Marshang; Ginger R. Dreger; Arnold & Porter LLP(57) **ABSTRACT**

The invention provides a method for more effective treatment of patients susceptible to or diagnosed with tumors overexpressing HER2, as determined by a gene amplification assay, with a HER2 antibody. Such method comprises administering a cancer-treating dose of the HER2 antibody, preferably in addition to chemotherapeutic agents, to a subject in whose tumor cells her2 has been found to be amplified e.g., by fluorescent in situ hybridization.

6 Claims, No Drawings

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US 8,076,066 B2

Page 7

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US 8,076,066 B2

1

GENE DETECTION ASSAY FOR IMPROVING THE LIKELIHOOD OF AN EFFECTIVE RESPONSE TO A HER2 ANTIBODY CANCER THERAPY

This continuation application claims priority to non-provisional application Ser. No. 09/863,101 filed May 18, 2001 now abandoned which claims priority under 35 U.S.C. §119 (e) of provisional application 60/205,754, filed May 19, 2000, which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention concerns the treatment of cancers characterized by the overexpression of a tumor antigen, such as an ErbB receptor, particularly HER2. More specifically, the invention concerns more effective treatment of human patients susceptible to or diagnosed with cancer, in which the tumor cells overexpress ErbB as determined by a gene amplification assay, with an ErbB antagonist, e.g., an anti-ErbB antibody. The invention further provides pharmaceutical packages for such treatment.

BACKGROUND OF THE INVENTION

Advancements in the understanding of genetics and developments in technology and epidemiology have allowed for the correlation of genetic abnormalities with certain malignancies as well as risk assessment of an individual for developing certain malignancies. However, most of the methodologies available for evaluation of tissue for the presence of genes associated with or predisposing an individual to a malignancy have well-known drawbacks. For example, methods that require disaggregation of the tissue, such as Southern, Northern, or Western blot analysis, are rendered less accurate by dilution of the malignant cells by the normal or otherwise non-malignant cells that are present in the same tissue. Furthermore, the resulting loss of tissue architecture precludes the ability to correlate malignant cells with the presence of genetic abnormalities in a context that allows morphological specificity. This issue is particularly problematic in tissue types known to be heterogeneous, such as in human breast carcinoma, where a significant percentage of the cells present in any area may be non-malignant.

The her2/neu gene encodes a protein product, often identified as p185HER2. The native p185HER2 protein is a membrane receptor-like molecule with homology to the epidermal growth factor receptor (EGFR). Amplification and overexpression of HER2 in human breast cancer has been correlated with shorter disease-free interval and shorter overall survival in some studies (van de Vijver et al. *New Eng. J. Med.* 317: 1239 (1988); Walker et al. *Br. J. Cancer* 60:426 (1989); Tandon et al. *J. Clin. Invest.* 7:1120 (1989); Wright et al. *Cancer Res.* 49:2087 (1989); McCann et al. *Cancer Res* 51:3296 (1991); Paterson et al. *Cancer Res.* 51:556 (1991); and Winstanley et al. *Br. J. Cancer* 63:447 (1991)) but not in others (Zhou et al. *Oncogene* 4:105 (1989); Heintz et al. *Arch Path Lab Med* 114:160 (1990); Kury et al. *Eur. J. Cancer* 26:946 (1990); Clark et al. *Cancer Res.* 51:944 (1991); and Ravdin et al. *J. Clin. Oncol.* 12:467-74 (1994)).

In an initial evaluation of 103 patients with breast cancer, those having more than three tumor cell positive axillary lymph nodes (node positive) were more likely to overexpress HER2 protein than patients with less than three positive nodes (Slamon et al. *Science* 235:177 (1987)). In a subsequent evaluation of 86 node-positive patients with breast cancer, there was a significant correlation among the extent of gene

2

amplification, early relapse, and short survival. HER2 overexpression was determined using Southern and Northern blotting, which correlate with the HER2 oncoprotein expression evaluated by Western blotting and immunohistochemistry (IHC) (Slamon et al. *Science* 235:177 (1987); Slamon et al. *Science* 244:707 (1989)). The median period of survival was found to be approximately 5-fold shorter in patients with more than five copies of the her2 gene than in patients without gene amplification. This correlation was present even after correcting for nodal status and other prognostic factors in multivariate analyses. These studies were extended in 187 node-positive patients and indicated that gene amplification, increased amounts of mRNA (determined by Northern blotting), and increased protein expression (determined immunohistochemically) were also correlated with shortened survival time (Slamon et al. *Science* 244:707 (1989)); (see also U.S. Pat. No. 4,968,603). Nelson et al. have compared her2/neu gene amplification using FISH with immunohistochemically determined overexpression in breast cancer (Nelson et al. *Modern Pathology* 9 (1) 21A (1996)).

Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing alteration of proteins in a heterogeneous tissue. Immunohistochemistry (IHC) techniques utilize an antibody to probe and visualize cellular antigens in situ, generally by chromagenic or fluorescent methods. This technique excels because it avoids the unwanted effects of disaggregation and allows for evaluation of individual cells in the context of morphology. In addition, the target protein is not altered by the freezing process.

However, in the clinical trial assay (CTA), IHC of formaldehyde-fixed, paraffin embedded tissue samples only demonstrated 50%-80% sensitivity, relative to frozen IHC samples (Press, *Cancer Research* 54:2771 (1994)). Thus, IHC can lead to false negative results, excluding from treatment patients who might benefit from the treatment.

Fluorescence in situ hybridization (FISH) is a recently developed method for directly assessing the presence of genes in intact cells. FISH is an attractive means of evaluating paraffin-embedded tissue for the presence of malignancy because it provides for cell specificity, yet overcomes the cross-linking problems and other protein-altering effects caused by formalin fixation. FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology (see, e.g., Anastasi et al., *Blood* 77:2456-2462 (1991); Anastasi et al., *Blood* 79:1796-1801 (1992); Anastasi et al., *Blood* 81:1580-1585 (1993); van Lom et al., *Blood* 82:884-888 (1992); Wolman et al., *Diagnostic Molecular Pathology* 1(3): 192-199 (1992); Zitzelberger, *Journal of Pathology* 172:325-335 (1994)).

To date, there has been no correlation of her2 gene amplification with anti-HER2 antibody treatment outcome, only with disease prognosis. The standard assay has been IHC on formalin fixed, paraffin embedded samples. These samples, when scored as 3+ or 2+, identify patients who are likely to benefit from treatment with an anti-HER2 antibody, like HERCEPTIN®. The 3+ and 2+ scores correlate with her2 gene amplification, e.g., as tested by FISH. However, there remains a need for more effective identification of candidates for successful ErbB antagonist therapies, such as HERCEPTIN® treatment.

SUMMARY OF THE INVENTION

The invention advantageously provides a method for increasing likelihood of effectiveness of an ErbB antagonist cancer treatment. The method comprises administering a can-

US 8,076,066 B2

3

cer treating dose of the ErbB antagonist to a subject wherein an erbB gene in tumor cells in a tissue sample from the subject has been found to be amplified. Preferably the ErbB is HER2. In a specific embodiment, the method further comprises administering a cancer treating dose of a chemotherapeutic, particularly a taxol.

In a specific preferred embodiment, exemplified herein, the invention provides a method for increasing likelihood of effectiveness of an anti-HER2 antibody to treat cancer. This method comprises administering a cancer treating dose of the anti-HER2 antibody to the subject in whom a her2 gene in tumor cells in a tissue sample from the subject have been found to be amplified.

The unexpected clinical results underlying the invention, in which gene amplification proved to be a more effective indication of antibody-based tumor therapy than protein detection by immunohistochemistry, extends to tumor antigens in general. Thus, any anti-tumor-specific antigen based antibody therapy can have increased likelihood of success in patients who are found to have gene amplification of the gene encoding the tumor antigen.

A particular advantage of the invention is that it permits selection of patients for treatment who, based on immunohistochemical criteria, would be excluded. Thus, in a specific embodiment, the subject has been found to have an antigen level corresponding to a 0 or 1+ score for HER2 by immunohistochemistry on a formaldehyde-fixed tissue sample.

The invention further provides a pharmaceutical package comprising an ErbB antagonist for treating a cancer, and instructions to administer the ErbB antagonist to a subject if an erbB gene in tumor cells in a tissue sample from the subject is amplified. Preferably the ErbB antagonist is an anti-ErbB antibody, such as an anti-HER2 antibody. In a further aspect, the instructions also teach administering a cancer treating dose of a chemotherapeutic, e.g., a taxol. Such pharmaceutical packages, including the instructions for use, can be provided for any antibody-based therapeutic specific for a tumor-specific antigen.

DETAILED DESCRIPTION

The present invention advantageously permits treatment of patients who have a greater likelihood of responding to the treatment by administering therapeutic agents, i.e., anti-tumor antigen therapeutic antibodies or ErbB receptor antagonists, to patients who are found to have an amplified gene encoding such a tumor antigen or ErbB receptor protein. The invention is based, in part, on the unexpected discovery that her2 gene amplification, e.g., as detected by fluorescence in situ hybridization (FISH), although it correlates with HER2 expression as detected by immunohistochemistry (IHC), provides a more accurate basis for selecting patients for treatment because FISH status unexpectedly correlates better with response to treatment. This outcome was surprising in part because FISH status has about the same rate of correlation with a clinical trial assay (CTA) IHC assay as another IHC assay (HERCEPT®). Based on this observation, FISH would be expected to have a similar correlation with treatment response. This outcome also surprises because direct measurement of protein (by immunoassay) would be expected to provide a more accurate assessment of a cancer therapy targeted to the protein than an indirect measure of expression, like gene amplification.

Evaluation of patient groups and subgroups demonstrates the power of gene amplification analysis for selecting patients more likely to respond to treatment. IHC provides a score for HER2 expression on tumor cells: 0 (no expression) through

4

3+ (very high levels of expression). Clinical selection criteria exclude patients with 0 and 1+ scores and select patients with 2+ and 3+ scores. The data show that 14% of combined 2+/3+ patients respond to HERCEPTIN®, while 20% of FISH+ (amplified her2 gene) patients respond to HERCEPTIN®. The 3+ subgroup has a 17% response rate, which is very close to the FISH+ subjects' response rate. However, the 2+ subgroup has less than half the response rate of FISH+ subjects. Thus, gene amplification clearly differentiates large subpopulations within the 2+ subgroup, permitting more effective treatment for those who are FISH+, and quickly identifying patients for whom alternative treatment modalities are appropriate and should commence immediately.

Gene amplification analysis also identifies patients who are unnecessarily excluded because of anomalies in the IHC analysis, particularly when the tests are performed on formalin fixed, paraffin embedded samples (such sample processing can disrupt or destroy antibody epitopes on the HER2 protein, but has much less impact on gene amplification assays). As shown in the examples, a subset of 0 and 1+ subjects are FISH+. These patients are likely to respond to anti-HER2 antibody therapy, e.g., with HERCEPTIN®, although by IHC criteria they would be excluded from receiving this treatment.

Thus, the present invention advantageously permits inclusion of patients who are more likely to benefit from treatment but who, by standard IHC criteria, would be excluded from treatment. At the same time, the invention permits exclusion of patients who should promptly seek an alternative mode of treatment because the anti-tumor antigen therapy (i.e., ErbB antagonist or tumor antigen-specific therapeutic antibody) is not likely to succeed.

In short, the present invention is a powerful adjunct to IHC assays for target protein expression level-based selection of patients. It can also be employed on its own, i.e., without IHC, to provide initial screening and selection of patients. The invention significantly improves screening and selection for subjects to receive a cancer-treating dose of an anti-tumor antigen therapeutic antibody treatment, ErbB receptor antagonist treatment, and other treatment targeted to overexpressed tumor antigens (or tumor-specific antigens), resulting in an increased likelihood of benefit from such treatments.

In another aspect, the invention concerns an article of manufacture or package, comprising a container, a composition within the container comprising an ErbB antagonist, e.g., an anti-ErbB antibody (or other anti-tumor-specific antigen antibody), optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB receptor, and a package insert containing instructions to administer the antagonist to patients who have been found to have an amplified erbB gene.

Definitions

As used herein, an "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3, and ErbB4 receptors, as well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid

US 8,076,066 B2

5

sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

ErbB receptors are examples of tumor-specific antigens or tumor antigens. The term "tumor antigen" is used herein to refer to a protein that is expressed at a higher level on tumor cells compared to normal cells. Generally, the normal cells for comparison are of the same tissue type, particularly phenotype, as the tumor, or from which the tumor arose. A "tumor specific antigen" refers to an antigen expressed either preferentially or only on tumor cells. Examples of tumor-specific antigens include, in addition to the ErbB receptors, MART1/Melan A, gp-100, and tyrosinase (in melanoma); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin/MUC-1 (in breast, pancreas, colon, and prostate cancers); prostate specific antigen/PSA (in prostate cancer); and carcinoembryonic antigen/CEA (in colon, breast, and gastrointestinal cancers).

By "amplification" is meant the presence of one or more extra gene copies of erbB or other tumor antigen-encoding gene in a chromosome complement. Gene amplification can result in overexpression of protein, e.g., ErbB receptor protein. Gene amplification in cells from a tissue sample can be measured by many techniques, particularly Fluorescence in situ Hybridization (FISH), but also including and not limited to quantitative PCR, quantitative Southern hybridization, and the like.

By "tissue sample" is meant a collection of similar cells obtained from a tissue of a subject or patient, preferably containing nucleated cells with chromosomal material. The four main human tissues are (1) epithelium; (2) the connective tissues, including blood vessels, bone and cartilage; (3) muscle tissue; and (4) nerve tissue. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like. In one embodiment of the invention, the tissue sample is "non-hematologic tissue" (i.e., not blood or bone marrow tissue).

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample may be analyzed at both morphological and molecular levels, or may be analyzed with respect to both protein and nucleic acid.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis with the performance and/or results of a second analysis. For example, one may use the results of a first analysis in carrying out the second analysis and/or one may use the results of a first analysis to determine whether a second analysis should be performed and/or one may compare the results of a first analysis with the results of a second analysis. In relation to IHC combined with FISH, one may use the results of IHC to determine whether FISH should be performed and/or one may compare the level of protein expression with gene amplification to further characterize a tumor biopsy (e.g. to compare HER2 protein expression with her2 gene amplification).

6

One advantageous feature of the invention is the ability to identify patients likely to benefit from treatment using FISH even if IHC indicates that they are antigen low.

By "nucleic acid" is meant to include any DNA or RNA, for example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing an RNA (rRNA, tRNA, or mRNA, the latter capable of translation as a protein) or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); Transforming Growth Factor alpha (TGF-alpha) (Marquardt et al., Science 223: 1079-1082 (1984)); amphiregulin, also known as schwannoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al., Science 259:1604-1607 (1993); and Sasada et al. Biochem. Biophys. Res. Commun. 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939 (1991)); epiregulin (Toyoda et al. J. Biol. Chem. 270:7495-7500 (1995); and Komurasaki et al. Oncogene 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., Proc. Natl. Acad. Sci. 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al., J. Biol. Chem. 272 (6):3330-3335 (1997)). ErbB ligands that bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide comprising an amino acid sequence encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641, 869 or Marchionni et al., Nature, 362:312-318 (1993), and biologically active variants of such polypeptides. Examples of heregulins include heregulin-alpha, heregulin-beta1, heregulin-beta2, and heregulin-beta3 (Holmes et al., Science, 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69: 205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Cell 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., Nature, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. J. Biol. Chem. 270:14523-14532 (1995)); gamma-heregulin (Schaefer et al. Oncogene 15:1385-1394 (1997)). An example of a biologically active fragment/amino acid sequence variant of a native sequence HRG polypeptide, is an EGF-like domain fragment (e.g., HRG-beta1, 177-244).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be

US 8,076,066 B2

7

isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., (J. Biol. Chem., 269(20): 14661-14665 (1994)), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3, and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4, or EGFR. Other proteins, such as a cytokine receptor subunit (e.g., gp130), may be included in the hetero-oligomer.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. (Ann. Rev. Biochem. 56:881-914 (1987)), including variants thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (Proc. Natl. Acad. Sci. USA 87:4207-4211 (1990)). ErbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), Mab 455 (ATCC CRL HB 8507), Mab 225 (ATCC CRL 8508), Mab 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, PCT Publication No. WO 96/40210).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., (Proc. Natl. Acad. Sci. USA 82:6497-6501 (1985)) and Yamamoto et al. (Nature 319:230-234 (1986)) (Genebank accession number X03363), and variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat p185neu. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include Mabs 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB-12215) (see, U.S. Pat. No. 5,772,997; PCT Publication No. WO 98/17797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, and huMAb4D5-8 (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337, which is expressly incorporated herein by reference; and humanized 520C9 (PCT Publication No. WO 93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 and PCT Publication No. WO 97/00271.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968, as well as Kraus et al. (Proc. Natl. Acad. Sci. USA) 86:9193-9197 (1989)), including variants thereof. Exemplary antibodies that bind HER3 are described in U.S. Pat. No. 5,968,511, e.g., the 8B8 antibody (ATCC HB-12070) or a humanized variant thereof. The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in European Application No. EP 599,274; Plowman et al., (Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993)); and Plowman et al., (Nature, 366:473-475 (1993)), including variants thereof such as the HER4 isoforms disclosed in PCT Publication No. WO 99/19488.

An "ErbB antagonist" is any molecule that binds to an ErbB receptor and blocks ligand activation of the ErbB receptor. Such antagonists include, but are not limited to, modified ligands, ligand peptides (i.e., ligand fragments), soluble ErbB receptors, and, preferably, anti-ErbB antibodies.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

8

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the ErbB antagonist, e.g., anti-ErbB2 antibody, and more generally, any cancer in which administration of an antibody against an over-expressed antigen can treat the cancer. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount elicits antibody-mediated cytotoxicity, activates complement, has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time for disease progression (TTP), survival, tumor size, or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, melanoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small-cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface, such that an anti-ErbB antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I¹³¹, I¹²⁵, Y⁹⁰, and Re¹⁸⁶), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as ben-zodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibi-

otics such as aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carubicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, pectinomycin, pottiomycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitioestanol, mepitioestane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglutone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguanzone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procabazine; PSK®; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepe; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; carminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and anti-androgens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W B Saunders: Philadelphia, 1995),

especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

ErbB Receptor Tyrosine Kinases

The ErbB receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes at least four distinct members including Epidermal Growth Factor Receptor (EGFR or ErbB1), HER2 (ErbB2 or p 185neu), HER3 (ErbB3), and HER4 (ErbB4 or tyro2).

EGFR, encoded by the ErbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer, as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, Transforming Growth Factor alpha (TGF-alpha), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-alpha and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn, *supra*; Masui et al. *Cancer Research* 44: 1002-1007 (1984); and Wu et al. *J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the ErbB family, p185neu, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science*, 235:177-182(1987); Slamon et al., *Science*, 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder.

Antibodies directed against the rat p185neu and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185neu (see, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and WO94/22478). Drebin et al. (*Oncogene* 2:273-277(1988)) report that mixtures of antibodies reactive with two distinct regions of p185neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice (see also U.S. Pat. No. 5,824,311).

Hudziak et al., (*Mol. Cell. Biol.* 9(3):1165-1172 (1989)) describe the generation of a panel of anti-HER2 antibodies, which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5, which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-alpha (see, also, U.S. Pat. No. 5,677,171). The anti-HER2 antibodies discussed in Hudziak et al. were further characterized (Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):

59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9: 1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997)).

A recombinant humanized IgG1 version of the murine anti-HER2 antibody 4D5 (rhuMab HER2 or HERCEPTIN®; commercially available from Genentech, Inc., South San Francisco) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). HERCEPTIN® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. The current treatment protocol employs IHC to determine HER2 protein overexpression.

Other anti-HER2 antibodies with various properties have been described (Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., (*Proc. Natl. Acad. Sci. USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); PCT Publication No. WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; Klapper et al. *Oncogene* 14:2099-2109 (1997); and PCT Publication No. WO 98/17797).

Homology screening has resulted in the ErbB receptor family members: HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968; Kraus et al., *Proc. Natl. Acad. Sci. USA* 86:9193-9197 (1989)) and HER4 (European Patent Application No. EP 599 274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and Plowman et al., *Nature*, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al., *Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands: Epidermal Growth Factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, Heparin Binding Epidermal Growth Factor (HB-EGF), betacellulin, and epiregulin (Groenen et al. *Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta, and gamma heregulins (Holmes et al., *Science*, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al., *Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF) (for a review, see Groenen et al., *Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee et al. *Pharm. Rev.* 47:51-85 (1995)). Recently, two additional ErbB ligands were identified: neuregulin-2 (NRG-2), which is reported to bind either HER3 or HER4 (Chang et al., *Nature*: 387 509-512 (1997); and Carraway et al.

Nature 387:512-516 (1997)) and neuregulin-3, which binds HER4 (Zhang et al., (*Proc. Natl. Acad. Sci. USA*) 94(18): 9562-7 (1997)). HB-EGF, betacellulin, and epiregulin also bind to HER4.

While EGF and TGF-alpha do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase, (Earp et al., supra.) Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., *Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey et al., *Proc. Natl. Acad. Sci. USA* 92:1431-1435 (1995); and Lewis et al., *Cancer Res.*, 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8(1994)).

Detecting Gene Amplification

The present invention contemplates using any technique to detect gene amplification. (see, Boxer, *J. Clin. Pathol.* 53: 19-21(2000)). These techniques include in situ hybridization (Stoler, *Clin. Lab. Med.* 12:215-36 (1990), using radioisotope or fluorophore-labeled probes; polymerase chain reaction (PCR); quantitative Southern blotting, and other techniques for quantitating individual genes. Preferably probes or primers selected for gene amplification evaluation are highly specific, to avoid detecting closely related homologous genes.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. A hapten or epitope that is immunospecifically bound by an antibody can also serve as a label.

The term "fluorescently labeled nucleic acid probe" refers to a probe comprising (1) a nucleic acid having a sequence rendering it capable of hybridizing with a target nucleic acid sequence and (2) a fluorescent label. Preferably such hybridization is specific, i.e., it can occur under high stringency conditions.

Sample Preparation

Any tissue sample from a subject may be used. Examples of tissue samples that may be used include, but are not limited to, breast, prostate, ovary, colon, lung, endometrium, stomach, salivary gland or pancreas. The tissue sample can be obtained by a variety of procedures including, but not limited to surgical excision, aspiration, or biopsy. The tissue may be fresh or frozen. In one embodiment, the tissue sample is fixed and embedded in paraffin or the like.

The tissue sample may be fixed (i.e., preserved) by conventional methodology (See e.g., *Manual of Histological Staining Method of the Armed Forces Institute of Pathology*, 3rd Edition Lee G. Luna, HT (ASCP) Editor, The Blakston Division McGraw-Hill Book Company: New York; (1960); *The Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology* (1994) Ulreka V. Mikel, Editor, Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.). One of skill in the

US 8,076,066 B2

13

art will appreciate that the choice of a fixative is determined by the purpose for which the tissue is to be histologically stained or otherwise analyzed. One of skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin's or paraformaldehyde, may be used to fix a tissue sample.

Generally, the tissue sample is first fixed and is then dehydrated through an ascending series of alcohols, infiltrated, and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. By way of example, the tissue sample may be embedded and processed in paraffin by conventional methodology. Examples of paraffin that may be used include, but are not limited to, Paraplast, Broloid, and Tissuemay. Once the tissue sample is embedded, the sample may be sectioned by a microtome or the like. By way of example for this procedure, sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine, and the like. For example, the paraffin embedded sections may be attached to positively charged slides, slides coated with poly-L-lysine.

If paraffin has been used as the embedding material, the tissue sections are generally deparaffinized and rehydrated to water. The tissue sections may be deparaffinized by several conventional standard methodologies. For example, xylenes and a gradually descending series of alcohols may be used. Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Tex.) may be used.

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is generally carried out on cells or tissue sections fixed to slides. In situ hybridization may be performed by several conventional methodologies (see, e.g., Leitch et al., *In Situ Hybridization: A Practical Guide*, Oxford BIOS Scientific Publishers, Microscopy Handbooks v. 27 (1994)). In one in situ procedure, fluorescent dyes (such as fluorescein isothiocyanate (FITC) which fluoresces green when excited by an Argon ion laser) are used to label a nucleic acid sequence probe that is complementary to a target nucleotide sequence in the cell. Each cell containing the target nucleotide sequence will bind the labeled probe producing a fluorescent signal upon exposure, of the cells to a light source of a wavelength appropriate for excitation of the specific fluorochrome used. A "target nucleotide sequence" is a sequence specific for a over-expressed tumor antigen, such as ErbB. FISH analysis can be used in conjunction with other assays, including without limitation morphological staining (of serial sections or the same section; see PCT Publication No. WO 00/20641, specifically incorporated herein by reference).

Various degrees of hybridization stringency can be employed. As the hybridization conditions become more stringent, a greater degree of complementarity is required between the probe and target to form and maintain a stable duplex. Stringency is increased by raising temperature, lowering salt concentration, or raising formamide concentration. Adding dextran sulfate or raising its concentration may also increase the effective concentration of labeled probe to increase the rate of hybridization and ultimate signal intensity. After hybridization, slides are washed in a solution generally containing reagents similar to those found in the hybridization solution with washing time varying from minutes to hours depending on required stringency. Longer or more stringent washes typically lower nonspecific background but run the risk of decreasing overall sensitivity.

14

Probes used in the FISH analysis may be either RNA or DNA oligonucleotides or polynucleotides and may contain not only naturally occurring nucleotides but their analogs like digoxigenin dCTP, biotin dCTP 7-azaguanosine, azidothymidine, inosine, or uridine. Other useful probes include peptide probes and analogues thereof, branched gene DNA, peptidomimetics, peptide nucleic acid (PNA), and/or antibodies.

Probes should have sufficient complementarity to the target nucleic acid sequence of interest so that stable and specific binding occurs between the target nucleic acid sequence and the probe. The degree of homology required for stable hybridization varies with the stringency of the hybridization medium and/or wash medium. Preferably, completely homologous probes are employed in the present invention, but persons of skill in the art will readily appreciate that probes exhibiting lesser but sufficient homology can be used in the present invention (see e.g., Sambrook, J., et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Press, (1989)).

One of skill in the art will appreciate that the choice of probe depends on the characteristics of the target gene of interest. Examples of amplification include, but are not limited to, her2/neu in breast and ovarian cancer, n-myc in neuroblastoma, c-myc in small cell lung cancer. By way of example for evaluating her2/neu amplification a probe spanning a 140 kb region on the long arm of chromosome 17 containing the her2/neu gene (17q 11.2-17q12) may be used. A probe for the -satellite sequences in the centromeric region of chromosome 17(D17Z1) may be used to evaluate for aneuploidy of chromosome 17 as a source or cause for her2/neu amplification. For example, a cocktail version of these probes may be obtained from Vysis, Inc. where each probe is directly labeled with easily distinguishable fluorophores, such as SPECTRUM ORANGE® and SPECTRUM GREEN®.

Probes may also be generated and chosen by several means including, but not limited to, mapping by in situ hybridization, somatic cell hybrid panels, or spot blots of sorted chromosomes; chromosomal linkage analysis; or cloned and isolated from sorted chromosome libraries from human cell lines or somatic cell hybrids with human chromosomes, radiation somatic cell hybrids, microdissection of a chromosome region, or from yeast artificial chromosomes (YACs) identified by PCR primers specific for a unique chromosome locus or other suitable means like an adjacent YAC clone. Probes may be genomic DNA, cDNA, or RNA cloned in a plasmid, phage, cosmid, YAC, Bacterial Artificial Chromosomes (BACs), viral vector, or any other suitable vector. Probes may be cloned or synthesized chemically by conventional methods. When cloned, the isolated probe nucleic acid fragments are typically inserted into a vector, such as lambda phage, pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host (see, e.g., Sambrook, supra).

Probes are preferably labeled with a fluorophore. Examples of fluorophores include, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocrytherin, phycocyanin, or commercially available fluorophores such as SPECTRUM ORANGE® and SPECTRUM GREEN®, and/or derivatives of any one or more of the above. Multiple probes used in the assay may be labeled with more than one distinguishable fluorescent or pigment color. These color differences provide a means to identify the hybridization positions of specific probes. Moreover, probes that are not separated spatially can be identified by a different color light or pigment resulting from mixing two other colors (e.g., light red+green=yellow), pigment (e.g., blue+yellow=green), or by using a filter set that passes only one color at a time.

US 8,076,066 B2

15

Probes can be labeled directly or indirectly with the fluorophor, utilizing conventional methodology. Additional probes and colors may be added to refine and extend this general procedure to include more genetic abnormalities or serve as internal controls. By way of example the her2/neu gene is in chromosome 17, and as an internal control a probe for satellite sequences specific for chromosome 17 (D17Z1) may be used (Vysis, Inc.) to prove diploidy in areas of non-malignant cells and/or to establish the presence or absence of chromosome 17 aneuploidy in areas of her2/neu amplification.

After processing for FISH, the slides may be analyzed by standard techniques of fluorescence microscopy (see, e.g., Ploem and Tanke, *Introduction to Fluorescence Microscopy*, Oxford University Press: New York (1987)). Briefly, each slide is observed using a microscope equipped with appropriate excitation filters, dichromic, and barrier filters. Filters are chosen based on the excitation and emission spectra of the fluorochromes used. Photographs of the slides may be taken with the length of time of film exposure depending on the fluorescent label used, the signal intensity and the filter chosen. For FISH analysis the physical loci of the cells of interest determined in the morphological analysis are recalled and visually conformed as being the appropriate area for FISH quantification.

In order to correlate IHC with FISH, one may use a computer-driven, motorized stage which stores location of coordinates. This may be used to evaluate the same area by two different analytical techniques. For example, color images of the morphologically stained areas may be captured and saved using a computer-assisted cooled CCD camera. The same section may be subsequently taken through the FISH procedure, the stored locations recalled, and the designated areas scored for the presence of fluorescent nuclear signals. A similar procedure for IHC followed by FISH is contemplated.

Typically, hundreds of cells are scanned in a tissue sample and quantification of the specific target nucleic acid sequence is determined in the form of fluorescent spots, which are counted relative to the number of cells. Deviation of the number of spots in a cell from a norm (e.g., such as probing for the her2/neu gene in a normal cell will produce two copies, abnormal greater than two) is indicative of a greater likelihood of benefit from a tumor antigen-specific antibody therapy, e.g., an ErbB antagonist therapy. As exemplified infra, her2 gene amplification provides a much more effective indication of the likelihood that an anti-HER2 antibody therapy will be effective.

Pharmaceutical Formulations

Therapeutic formulations of the antagonists, e.g., antibodies, used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohy-

16

drates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICTM or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, vascular endothelial factor (VEGF), or an antibody that binds to a different epitope on the target ErbB, in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, a chemotherapeutic, a cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.

The formulations to be used for in vivo administration are preferably, and in the case of humans, must be, sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Treatment with the Anti-ErbB Antagonists

It is contemplated that, according to the present invention, the anti-ErbB antibodies or other antagonists may be used to treat various conditions characterized by overexpression and/or activation of the ErbB receptor in patients who have been found to have an amplified erbB gene. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal,

liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and any other active agents of the invention are administered to a human patient in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB antibody and a chemotherapeutic agent, e.g., a taxoid. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells (tumor resection) and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antagonist, e.g., antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Pharmaceutical Packages: Articles of Manufacture

In a related aspect of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, optionally labeled, and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a

composition that is effective for treating the condition and preferably has a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-tumor antigen therapeutic antibody or an ErbB antagonist, e.g., an anti-ErbB antibody. A label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. This second buffer can be used to reconstitute the active agent, if that is provided as a lyophilized or dried powder, or to dilute a concentrated preparation of the active agent. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In addition, the article of manufacture comprises a package insert or inserts with instructions for use in patients who have been found to have erbB gene amplification, e.g., by FISH testing. Such patients may be subjects who, by IHC, would be excluded from treatment with the ErbB antagonist, e.g., patients who score a 0 or 1+ using an anti-HER2 antibody.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

Further details of the invention are illustrated by the following non-limiting Examples.

EXAMPLE 1

Concordance Between the Clinical Trials Assay (CTA) and Fluorescence In Situ Hybridization (FISH) in the HERCEPTIN® Pivotal Trials

Overexpression of HER2 at the 2+ or 3+ level by immunohistochemistry (IHC) was required for enrollment in the pivotal HERCEPTIN® metastatic breast cancer trials. The Clinical Testing Assay (CTA) involves two separate IHC assays performed with either monoclonal antibodies 4D5 (after protease digestion of the formalin fixed sample) or CB11 (after heat treatment of the formalin fixed sample). Subjects were eligible if either assay was scored at 2+ or 3+. If both were performed, the final score was the higher of the two results.

Concordance between the CTA and another IHC, HERCEPTEST® (HT), is 79%. This was the basis for FDA approval of HT to aid in the selection of patients for HERCEPTIN® therapy.

This Example describes a similar concordance study, utilizing clinical material submitted for screening for the HERCEPTIN® pivotal trials, that compares the CTA to her2/neu gene amplification measured by the PathVysion FISH assay. In the pivotal trials, 5998 subjects were screened for HER2 expression; 1915 (32%) were positive by the CTA and 4083 (68%) were negative. A random sample of 623 specimens (1:1 ratio of positive:negative) were selected for this analysis, 317 CTA+ and 306 CTA-. Specimens were not freshly cut

US 8,076,066 B2

19

from blocks. They had been stored between 2 and 4 years as 4-6 μ sections on glass slides. Each section was assayed for her2/neu amplification using the protocol specified in the package insert of the PathVysion assay. Amplification was defined as a signal ratio of greater than or equal to 2. The results are shown in Table 1.

TABLE 1

FISH/CTA Concordance						
		CTA				
		0	1+	2+	3+	
FISH	-	207	28	67	21	
	+	7	2	21	176	
		4%	7%	24%	89%	529

FISH+ = HER2:CEP17 signal ratio ≥ 2

Concordance = 82% (79-85%)

For the total 623 specimens tested, a FISH signal result was obtained in 529. Assay failure occurred in 19.9% of CTA- and 10.4% CTA+ samples. Amplification in the 0, 1+, 2, and 3+ groups was 4.2%, 6.7%, 23.9%, and 89.3%, respectively. The sample concordance was 81.3%, similar to the CTA/HT concordance of 79%. Single copy overexpression was 31%, predominantly in the 2+ group. Amplification was rarely (4.6%) noted in the CTA- group. The higher assay failure rate in the CTA- group may be due to non-assay related factors such as tissue fixation. These may have also resulted in false negative results for IHC.

These data were closely interpreted to suggest that her2/neu amplification status may have unexpectedly superior predictive value for identifying patients who are more likely to benefit from HERCEPTIN® treatment as compared to HERCEPTIN®. The observation that only 24% of 2+ patients are FISH+ suggest that this sub-group may have less predictable treatment outcomes when selected by IHC only. Identification of FISH+ patients in the 1+ and 0 sub-groups might identify subjects who, though failing the IHC criteria for HERCEPTIN® treatment, would likely benefit from HERCEPTIN® treatment. A direct analysis of HERCEPTIN® benefit based on FISH score compared to IHC score is presented in Example 2.

EXAMPLE 2

FISH/Clinical Outcome Study

This example links the results from three HERCEPTIN® Trials with FISH status. In this study, 805 subjects were selected at random from all three trials. Of these, 167 lacked slides. Another 78 assays (9.7%) failed. Thus, formalin-fixed cut sections stored between 2.5 and 4.5 years from 540 subjects provided the sample pool for this study. There were no imbalances in demographics or prognostic indicators in these samples. Results are reported for different treatment groups.

Correlation of FISH status with response was evaluated for patients who received HERCEPTIN® as a second or third line therapy. These data are reported for 2+ and 3+ (by CTA) subjects in Table 2.

20

TABLE 2

FISH/Response with single agent HERCEPTIN®, 2nd or 3rd line Therapy, 2+/3+ Combined		
	FISH+	FISH-
Response	21	0
No response	84	37
response rate	20% (12.5-27.5%)	0% (0.7%)

N = 142

The 20% response rate of FISH+ subjects unexpectedly exceeds the 15% response rate of 2+ and 3+ patients in this study and 14% response rate observed in patients selected by CTA with a 2+ or 3+ immunohistochemistry score during the pivotal trials. Thus, while FISH correlates well with IHC to about the same degree as another IHC assay, the Hercep Test, as shown in Example 1, it unexpectedly is superior in identifying patients who are more likely to benefit from HERCEPTIN® therapy.

When these data were broken down into the components 3+ and 2+ subjects, the same 20% response rate of FISH+ subjects was seen (Tables 3 and 4).

TABLE 3

FISH/Response with single agent HERCEPTIN®, 2nd or 3rd line therapy, 3+ subgroup		
	FISH+	FISH-
Response	18	0
No response	72	17
response rate	20% (12-28%)	0% (0-14%)

N = 107

TABLE 4

FISH/Response with single agent HERCEPTIN®, 2nd or 3rd line therapy, 2+ subgroup		
	FISH+	FISH-
Response	3	0
No response	12	20
response rate	20% (1-40%)	0% (0-14%)

N = 35

In the 3+ sub-group, the FISH+ response rate (20%) was very close but still exceeded the 17% response rate of 3+ subjects. The 2+ subgroup showed a much greater difference, with only a 9% response rate versus 20% by FISH+ selection. These data show that FISH+ status (her2 gene amplification) greatly increases the likelihood of response to HERCEPTIN®.

Data were also evaluated for patient responses to HERCEPTIN® as a first line therapy (Table 5).

TABLE 5

FISH/Response with single agent HERCEPTIN® as 1st line therapy, 2+/3+ combined		
	FISH+	FISH-
Response	17	1
No response	24	20
response rate	41% (26-56%)	20% (0-14%)

N = 62

US 8,076,066 B2

21

The 41% response rate of FISH+ subjects was notably greater than the 27% response rate of 3+, 2+ subjects.

The surprising increase in likelihood of beneficial response based on FISH analysis extended to responses to chemotherapy plus HERCEPTIN®, as shown in Table 6. FISH+ subjects showed a much greater response to chemotherapy and HERCEPTIN® (54%) than FISH- (41%). Tables 7-9 contain more extensive data, broken down by different chemotherapeutic agents (adriamycin and cyclophosphamide, AC; and Paclitaxel, P) and different endpoints (response rate, time to progression, and survival) for HERCEPTIN® in combination with chemotherapy.

TABLE 6

FISH/Response rate to chemotherapy +/- HERCEPTIN®, 1st line therapy; 2+/3+ combined		
	C alone	C + H
FISH-	39% (26-52%)	41% (27-55%)
FISH+	27% (19-35%)	54% (45-63%)

N = 336

TABLE 7

Response rate of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	56*	42	41*	17	50*	32
3+	349	60*	42	49*	17	56*	31
FISH+	240	58*	40	49*	14	54*	27

*p < 0.05

TABLE 8

Time to progression (months) of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	7.8*	6.1	6.9*	2.7	7.4*	4.6
3+	349	8.1*	6.0	7.1*	3.0	7.8*	4.6
FISH+	240	7.8*	6.2	7.0*	3.2	7.3*	4.6

*p < 0.05

TABLE 9

Survival (months) of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	27	21	22	18	25*	20
3+	349	31*	21	25	18	29*	20
FISH+	240	29*	20	25*	14	27*	18

*p < 0.05

These data uniformly confirm that FISH+ analysis, though correlating closely to IHC, provides a much more accurate indicator of likelihood of success with HERCEPTIN® treatment. Across the board, FISH+ selection has about 1/3 (30%) greater response rate than 2+/3+ IHC-selection. When focused on 2+ patients, FISH status provides a much more effective tool for patient selection. FISH states also identifies patients who, because of 0 or 1+ status as determined by IHC, would otherwise be excluded from treatment.

22

These observations have broad implications for ErbB receptor antagonist-based cancer therapies and anti-tumor antigen cancer therapies in general. Thus erbB antagonists, e.g., anti-erbB receptor antibodies like HERCEPTIN®, can have an increased likelihood of efficacy when administered to patients who are positive for erbB gene amplification, e.g., by a FISH test. This is certainly the case, based on these data, with HERCEPTIN®.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed:

1. A method of identifying and treating a breast cancer patient disposed to respond favorably to a HER2 antibody, huMAb4D5-8, which method comprises detecting her2 gene

amplification in cancer cells in a breast tissue sample from the patient and treating the patient with her2 gene amplification with the HER2 antibody in an amount effective to treat the breast cancer, wherein the patient's cancer cells express HER2 at a 0 or 1+ level by immunohistochemistry.

2. The method of claim 1 wherein her2 gene amplification is detected by detecting fluorescence of a fluorescent-labeled nucleic acid probe hybridized to the gene.

US 8,076,066 B2

23

3. The method of claim 1 wherein a formaldehyde-fixed tissue sample containing the patient's breast cancer cells has been subjected to immunohistochemistry assay and found to express HER2 at a 0 or 1+ level.

4. The method of claim 3 wherein the immunohistochemistry assay is performed prior to detecting her2 gene amplification. ⁵

24

5. The method of claim 1 which further comprises administering a cancer treating dose of a chemotherapeutic drug.

6. The method of claim 5 wherein the chemotherapeutic drug is a taxoid.

* * * * *

EXHIBIT H



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(12) **United States Patent
Mass**(10) **Patent No.:** **US 8,440,402 B2**
(45) **Date of Patent:** ***May 14, 2013**(54) **GENE DETECTION ASSAY FOR IMPROVING
THE LIKELIHOOD OF AN EFFECTIVE
RESPONSE TO A HER2 ANTIBODY CANCER
THERAPY**(75) Inventor: **Robert D. Mass**, Mill Valley, CA (US)(73) Assignee: **Genentech, Inc.**, South San Francisco,
CA (US)(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.This patent is subject to a terminal dis-
claimer.(21) Appl. No.: **13/323,322**(22) Filed: **Dec. 12, 2011**(65) **Prior Publication Data**

US 2012/0093838 A1 Apr. 19, 2012

Related U.S. Application Data(63) Continuation of application No. 11/690,304, filed on
Mar. 23, 2007, now Pat. No. 8,076,066, which is a
continuation of application No. 09/863,101, filed on
May 18, 2001, now abandoned.(60) Provisional application No. 60/205,754, filed on May
19, 2000.(51) **Int. Cl.****C12Q 1/68** (2006.01)
G01N 33/574 (2006.01)(52) **U.S. Cl.**USPC **435/6.1; 435/7.23**(58) **Field of Classification Search** None
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**

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Primary Examiner — Laura B Goddard(74) *Attorney, Agent, or Firm* — Diane Marschang; Ginger
R. Dreger; Arnold & Porter LLP(57) **ABSTRACT**

The invention provides a method for more effective treatment of patients susceptible to or diagnosed with tumors overexpressing HER2, as determined by a gene amplification assay, with a HER2 antibody. Such method comprises administering a cancer-treating dose of the HER2 antibody, preferably in addition to chemotherapeutic agents, to a subject in whose tumor cells her2 has been found to be amplified e.g., by fluorescent in situ hybridization.

6 Claims, No Drawings

US 8,440,402 B2

Page 2

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US 8,440,402 B2

1

GENE DETECTION ASSAY FOR IMPROVING THE LIKELIHOOD OF AN EFFECTIVE RESPONSE TO A HER2 ANTIBODY CANCER THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 11/690,304, filed Mar. 23, 2007 now U.S. Pat. No. 8,076,066, which is a continuation application of and claims priority to U.S. patent application Ser. No. 09/863,101 filed May 18, 2001 (abandoned) which claims priority under 35 U.S.C. §119(e) of provisional application 60/205,754, filed May 19, 2000, all of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The present invention concerns the treatment of cancers characterized by the overexpression of a tumor antigen, such as an ErbB receptor, particularly HER2. More specifically, the invention concerns more effective treatment of human patients susceptible to or diagnosed with cancer, in which the tumor cells overexpress ErbB as determined by a gene amplification assay, with an ErbB antagonist, e.g., an anti-ErbB antibody. The invention further provides pharmaceutical packages for such treatment.

BACKGROUND OF THE INVENTION

Advancements in the understanding of genetics and developments in technology and epidemiology have allowed for the correlation of genetic abnormalities with certain malignancies as well as risk assessment of an individual for developing certain malignancies. However, most of the methodologies available for evaluation of tissue for the presence of genes associated with or predisposing an individual to a malignancy have well-known drawbacks. For example, methods that require disaggregation of the tissue, such as Southern, Northern, or Western blot analysis, are rendered less accurate by dilution of the malignant cells by the normal or otherwise non-malignant cells that are present in the same tissue. Furthermore, the resulting loss of tissue architecture precludes the ability to correlate malignant cells with the presence of genetic abnormalities in a context that allows morphological specificity. This issue is particularly problematic in tissue types known to be heterogeneous, such as in human breast carcinoma, where a significant percentage of the cells present in any area may be non-malignant.

The her2/neu gene encodes a protein product, often identified as p185HER2. The native p185HER2 protein is a membrane receptor-like molecule with homology to the epidermal growth factor receptor (EGFR). Amplification and overexpression of HER2 in human breast cancer has been correlated with shorter disease-free interval and shorter overall survival in some studies (van de Vijver et al. *New Eng. J. Med.* 317: 1239 (1988); Walker et al. *Br. J. Cancer* 60:426 (1989); Tandon et al. *J. Clin. Invest.* 7:1120 (1989); Wright et al. *Cancer Res.* 49:2087 (1989); McCann et al. *Cancer Res.* 51:3296 (1991); Paterson et al. *Cancer Res.* 51:556 (1991); and Winstonley et al. *Br. J. Cancer* 63:447 (1991)) but not in others (Zhou et al. *Oncogene* 4:105 (1989); Heintz et al. *Arch Path Lab Med* 114:160 (1990); Kury et al. *Eur. J. Cancer* 26:946 (1990); Clark et al. *Cancer Res.* 51:944 (1991); and Ravdin et al. *J. Clin. Oncol.* 12:467-74 (1994)).

2

In an initial evaluation of 103 patients with breast cancer, those having more than three tumor cell positive axillary lymph nodes (node positive) were more likely to overexpress HER2 protein than patients with less than three positive nodes (Slamon et al. *Science* 235:177 (1987)). In a subsequent evaluation of 86 node-positive patients with breast cancer, there was a significant correlation among the extent of gene amplification, early relapse, and short survival. HER2 overexpression was determined using Southern and Northern blotting, which correlate with the HER2 oncoprotein expression evaluated by Western blotting and immunohistochemistry (IHC) (Slamon et al. *Science* 235:177 (1987); Slamon et al. *Science* 244:707 (1989)). The median period of survival was found to be approximately 5-fold shorter in patients with more than five copies of the her2 gene than in patients without gene amplification. This correlation was present even after correcting for nodal status and other prognostic factors in multivariate analyses. These studies were extended in 187 node-positive patients and indicated that gene amplification, increased amounts of mRNA (determined by Northern blotting), and increased protein expression (determined immunohistochemically) were also correlated with shortened survival time (Slamon et al. *Science* 244:707 (1989)); (see also U.S. Pat. No. 4,968,603). Nelson et al. have compared her2/neu gene amplification using FISH with immunohistochemically determined overexpression in breast cancer (Nelson et al. *Modern Pathology* 9 (1) 21A (1996)).

Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing alteration of proteins in a heterogeneous tissue. Immunohistochemistry (IHC) techniques utilize an antibody to probe and visualize cellular antigens in situ, generally by chromagenic or fluorescent methods. This technique excels because it avoids the unwanted effects of disaggregation and allows for evaluation of individual cells in the context of morphology. In addition, the target protein is not altered by the freezing process.

However, in the clinical trial assay (CTA), IHC of formaldehyde-fixed, paraffin embedded tissue samples only demonstrated 50%-80% sensitivity, relative to frozen IHC samples (Press, *Cancer Research* 54:2771 (1994)). Thus, IHC can lead to false negative results, excluding from treatment patients who might benefit from the treatment.

Fluorescence in situ hybridization (FISH) is a recently developed method for directly assessing the presence of genes in intact cells. FISH is an attractive means of evaluating paraffin-embedded tissue for the presence of malignancy because it provides for cell specificity, yet overcomes the cross-linking problems and other protein-altering effects caused by formalin fixation. FISH has historically been combined with classical staining methodologies in an attempt to correlate genetic abnormalities with cellular morphology (see, e.g., Anastasi et al., *Blood* 77:2456-2462 (1991); Anastasi et al., *Blood* 79:1796-1801 (1992); Anastasi et al., *Blood* 81:1580-1585 (1993); van Lom et al., *Blood* 82:884-888 (1992); Wolman et al., *Diagnostic Molecular Pathology* 1(3): 192-199 (1992); Zitzelberger, *Journal of Pathology* 172:325-335 (1994)).

To date, there has been no correlation of her2 gene amplification with anti-HER2 antibody treatment outcome, only with disease prognosis. The standard assay has been IHC on formalin fixed, paraffin embedded samples. These samples, when scored as 3+ or 2+, identify patients who are likely to benefit from treatment with an anti-HER2 antibody, like HERCEPTIN®. The 3+ and 2+ scores correlate with her2 gene amplification, e.g., as tested by FISH. However, there

US 8,440,402 B2

3

remains a need for more effective identification of candidates for successful ErbB antagonist therapies, such as HERCEPTIN® treatment.

SUMMARY OF THE INVENTION

The invention advantageously provides a method for increasing likelihood of effectiveness of an ErbB antagonist cancer treatment. The method comprises administering a cancer treating dose of the ErbB antagonist to a subject wherein an erbB gene in tumor cells in a tissue sample from the subject has been found to be amplified. Preferably the ErbB is HER2. In a specific embodiment, the method further comprises administering a cancer treating dose of a chemotherapeutic, particularly a taxol.

In a specific preferred embodiment, exemplified herein, the invention provides a method for increasing likelihood of effectiveness of an anti-HER2 antibody to treat cancer. This method comprises administering a cancer treating dose of the anti-HER2 antibody to the subject in whom a her2 gene in tumor cells in a tissue sample from the subject have been found to be amplified.

The unexpected clinical results underlying the invention, in which gene amplification proved to be a more effective indication of antibody-based tumor therapy than protein detection by immunohistochemistry, extends to tumor antigens in general. Thus, any anti-tumor-specific antigen based antibody therapy can have increased likelihood of success in patients who are found to have gene amplification of the gene encoding the tumor antigen.

A particular advantage of the invention is that it permits selection of patients for treatment who, based on immunohistochemical criteria, would be excluded. Thus, in a specific embodiment, the subject has been found to have an antigen level corresponding to a 0 or 1+ score for HER2 by immunohistochemistry on a formaldehyde-fixed tissue sample.

The invention further provides a pharmaceutical package comprising an ErbB antagonist for treating a cancer, and instructions to administer the ErbB antagonist to a subject if an erbB gene in tumor cells in a tissue sample from the subject is amplified. Preferably the ErbB antagonist is an anti-ErbB antibody, such as an anti-HER2 antibody. In a further aspect, the instructions also teach administering a cancer treating dose of a chemotherapeutic, e.g., a taxol. Such pharmaceutical packages, including the instructions for use, can be provided for any antibody-based therapeutic specific for a tumor-specific antigen.

DETAILED DESCRIPTION

The present invention advantageously permits treatment of patients who have a greater likelihood of responding to the treatment by administering therapeutic agents, i.e., anti-tumor antigen therapeutic antibodies or ErbB receptor antagonists, to patients who are found to have an amplified gene encoding such a tumor antigen or ErbB receptor protein. The invention is based, in part, on the unexpected discovery that her2 gene amplification, e.g., as detected by fluorescence in situ hybridization (FISH), although it correlates with HER2 expression as detected by immunohistochemistry (NC), provides a more accurate basis for selecting patients for treatment because FISH status unexpectedly correlates better with response to treatment. This outcome was surprising in part because FISH status has about the same rate of correlation with a clinical trial assay (CTA) NC assay as another NC assay (HERCEPTIN®). Based on this observation, FISH would be expected to have a similar correlation with treat-

4

ment response. This outcome also surprises because direct measurement of protein (by immunoassay) would be expected to provide a more accurate assessment of a cancer therapy targeted to the protein than an indirect measure of expression, like gene amplification.

Evaluation of patient groups and subgroups demonstrates the power of gene amplification analysis for selecting patients more likely to respond to treatment. NC provides a score for HER2 expression on tumor cells: 0 (no expression) through 3+ (very high levels of expression). Clinical selection criteria exclude patients with 0 and 1+ scores and select patients with 2+ and 3+ scores. The data show that 14% of combined 2+/3+ patients respond to HERCEPTIN®, while 20% of FISH+ (amplified her2 gene) patients respond to HERCEPTIN®. The 3+ subgroup has a 17% response rate, which is very close to the FISH+ subjects' response rate. However, the 2+ subgroup has less than half the response rate of FISH+ subjects. Thus, gene amplification clearly differentiates large subpopulations within the 2+ subgroup, permitting more effective treatment for those who are FISH+, and quickly identifying patients for whom alternative treatment modalities are appropriate and should commence immediately.

Gene amplification analysis also identifies patients who are unnecessarily excluded because of anomalies in the IHC analysis, particularly when the tests are performed on formalin fixed, paraffin embedded samples (such sample processing can disrupt or destroy antibody epitopes on the HER2 protein, but has much less impact on gene amplification assays). As shown in the examples, a subset of 0 and 1+ subjects are FISH+. These patients are likely to respond to anti-HER2 antibody therapy, e.g., with HERCEPTIN®, although by IHC criteria they would be excluded from receiving this treatment.

Thus, the present invention advantageously permits inclusion of patients who are more likely to benefit from treatment but who, by standard IHC criteria, would be excluded from treatment. At the same time, the invention permits exclusion of patients who should promptly seek an alternative mode of treatment because the anti-tumor antigen therapy (i.e., ErbB antagonist or tumor antigen-specific therapeutic antibody) is not likely to succeed.

In short, the present invention is a powerful adjunct to INC assays for target protein expression level-based selection of patients. It can also be employed on its own, i.e., without IHC, to provide initial screening and selection of patients. The invention significantly improves screening and selection for subjects to receive a cancer-treating dose of an anti-tumor antigen therapeutic antibody treatment, ErbB receptor antagonist treatment, and other treatment targeted to overexpressed tumor antigens (or tumor-specific antigens), resulting in an increased likelihood of benefit from such treatments.

In another aspect, the invention concerns an article of manufacture or package, comprising a container, a composition within the container comprising an ErbB antagonist, e.g., an anti-ErbB antibody (or other anti-tumor-specific antigen antibody), optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of ErbB receptor, and a package insert containing instructions to administer the antagonist to patients who have been found to have an amplified erbB gene.

DEFINITIONS

As used herein, an "ErbB receptor" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes EGFR, HER2, ErbB3, and ErbB4 receptors, as

US 8,440,402 B2

5

well as TEGFR (U.S. Pat. No. 5,708,156) and other members of this family to be identified in the future. The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a native sequence ErbB receptor or an amino acid sequence variant thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

ErbB receptors are examples of tumor-specific antigens or tumor antigens. The term "tumor antigen" is used herein to refer to a protein that is expressed at a higher level on tumor cells compared to normal cells. Generally, the normal cells for comparison are of the same tissue type, particularly phenotype, as the tumor, or from which the tumor arose. A "tumor specific antigen" refers to an antigen expressed either preferentially or only on tumor cells. Examples of tumor-specific antigens include, in addition to the ErbB receptors, MART1/Melan A, gp-100, and tyrosinase (in melanoma); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin/MUC-1 (in breast, pancreas, colon, and prostate cancers); prostate specific antigen PSA (in prostate cancer); and camuinoembryonic antigen/CEA (in colon, breast, and gastrointestinal cancers).

By "amplification" is meant the presence of one or more extra gene copies of erbB or other tumor antigen-encoding gene in a chromosome complement. Gene amplification can result in overexpression of protein, e.g., ErbB receptor protein. Gene amplification in cells from a tissue sample can be measured by many techniques, particularly Fluorescence in situ Hybridization (FISH), but also including and not limited to quantitative PCR, quantitative Southern hybridization, and the like.

By "tissue sample" is meant a collection of similar cells obtained from a tissue of a subject or patient, preferably containing nucleated cells with chromosomal material. The four main human tissues are (1) epithelium; (2) the connective tissues, including blood vessels, bone and cartilage; (3) muscle tissue; and (4) nerve tissue. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like. In one embodiment of the invention, the tissue sample is "non-hematologic tissue" (i.e., not blood or bone marrow tissue).

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, e.g., a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample may be analyzed at both morphological and molecular levels, or may be analyzed with respect to both protein and nucleic acid.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis with the performance and/or results of a second analysis. For example, one may use the results of a first analysis in carrying out the second analysis and/or one may use the results of a first

6

analysis to determine whether a second analysis should be performed and/or one may compare the results of a first analysis with the results of a second analysis. In relation to IHC combined with FISH, one may use the results of INC to determine whether FISH should be performed and/or one may compare the level of protein expression with gene amplification to further characterize a tumor biopsy (e.g. to compare HER2 protein expression with her2 gene amplification). One advantageous feature of the invention is the ability to identify patients likely to benefit from treatment using FISH even if IHC indicates that they are antigen low.

By "nucleic acid" is meant to include any DNA or RNA, for example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing an RNA (rRNA, tRNA, or mRNA, the latter capable of translation as a protein) or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); Transforming Growth Factor alpha (TGF-alpha) (Marquardt et al., Science 223: 1079-1082 (1984)); amphiregulin, also known as schwannoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al., Science 259:1604-1607 (1993); and Sasada et al. Biochem. Biophys. Res. Commun. 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939 (1991)); epiregulin (Toyoda et al., J. Biol. Chem. 270:7495-7500 (1995); and Komurasaki et al. Oncogene 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., Nature 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., Proc. Natl. Acad. Sci. 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al., J. Biol. Chem. 272 (6):3330-3335 (1997)). ErbB ligands that bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide comprising an amino acid sequence encoded by the heregulin gene product as disclosed in U.S. Pat. No. 5,641,869 or Marchionni et al., Nature, 362:312-318 (1993), and biologically active variants of such polypeptides. Examples of heregulins include heregulin-alpha, heregulin-beta1, heregulin-beta2, and heregulin-beta3 (Holmes et al., Science, 256:1205-1210 (1992); and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69: 205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Cell 1172:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al., Nature, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. J. Biol. Chem. 270:14523-14532 (1995)); gamma-heregulin

US 8,440,402 B2

7

(Schaefer et al. *Oncogene* 15:1385-1394 (1997)). An example of a biologically active fragment/amino acid sequence variant of a native sequence HRG polypeptide, is an EGF-like domain fragment (e.g., HRG-beta1, 177-244).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., (*J. Biol. Chem.*, 269(20):14661-14665 (1994)), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3, and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4, or EGFR. Other proteins, such as a cytokine receptor subunit (e.g., gp130), may be included in the hetero-oligomer.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. (*Ann. Rev. Biochem.* 56:881-914 (1987)), including variants thereof (e.g., a deletion mutant EGFR as in Humphrey et al., (*Proc. Natl. Acad. Sci. USA* 87:4207-4211 (1990)). ErbB1 refers to the gene encoding the EGFR protein product. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), Mab 455 (ATCC CRL HB 8507), MAb 225 (ATCC CRL 8508), Mab 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533) and variants thereof, such as chimerized 225 (C225) and reshaped human 225 (H225) (see, PCT Publication No. WO 96/40210).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., (*Proc. Natl. Acad. Sci. USA* 82:6497-6501 (1985)) and Yamamoto et al. (*Nature* 319:230-234 (1986)) (Genebank accession number X03363), and variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat p185neu. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include MAb 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB-12215) (see, U.S. Pat. No. 5,772,997; PCT Publication No. WO 98/17797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, and huMAb4D5-8 (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337, which is expressly incorporated herein by reference; and humanized 520C9 (PCT Publication No. WO 93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 and PCT Publication No. WO 97/00271.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968, as well as Kraus et al. (*Proc. Natl. Acad. Sci. USA* 86:9193-9197 (1989)), including variants thereof. Exemplary antibodies that bind HER3 are described in U.S. Pat. No. 5,968,511, e.g., the 8B8 antibody (ATCC BIB-12070) or a humanized variant thereof. The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in European Application No. EP 599,274; Plowman et al., (*Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993)); and Plowman et al., (*Nature*, 366:473-475 (1993)), including variants thereof such as the HER4 isoforms disclosed in PCT Publication No. WO 99/19488.

An "ErbB antagonist" is any molecule that binds to an ErbB receptor and blocks ligand activation of the ErbB recep-

8

tor. Such antagonists include, but are not limited to, modified ligands, ligand peptides (i.e., ligand fragments), soluble ErbB receptors, and, preferably, anti-ErbB antibodies.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

A "disorder" is any condition that would benefit from treatment with the ErbB antagonist, e.g., anti-ErbB2 antibody, and more generally, any cancer in which administration of an antibody against an over-expressed antigen can treat the cancer. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies; neuronal, glial, astrocytic, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoele disorders; and inflammatory, angiogenic and immunologic disorders.

The term "therapeutically effective amount" is used to refer to an amount having antiproliferative effect. Preferably, the therapeutically effective amount elicits antibody-mediated cytotoxicity, activates complement, has apoptotic activity, or is capable of inducing cell death, and preferably death of benign or malignant tumor cells, in particular cancer cells. Efficacy can be measured in conventional ways, depending on the condition to be treated. For cancer therapy, efficacy can, for example, be measured by assessing the time for disease progression (TTP), survival, tumor size, or determining the response rates (RR) (see the Example below).

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, melanoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small-cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, and various types of head and neck cancer.

An "ErbB-expressing cancer" is one comprising cells which have ErbB protein present at their cell surface, such that an anti-ErbB antibody is able to bind to the cancer.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I¹³¹, I¹²⁵, Y⁹⁰, and Re¹⁸⁶), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as ben-zodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide,

US 8,440,402 B2

9

triethylenethiophosphoramidate and trimethylolmelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, pepomycin, potfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; antimetabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, flouxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglutone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguanzone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procabazine; PSK®; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepea; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; caminomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone; and anti-androgens such as flutamide and nilutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB-overexpressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), TAXOL®, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents

10

such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

ErbB Receptor Tyrosine Kinases

The ErbB receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes at least four distinct members including Epidermal Growth Factor Receptor (EGFR or ErbB1), HER2 (ErbB2 or p185neu), HER3 (ErbB3), and HER4 (ErbB4 or tyro2).

EGFR, encoded by the ErbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer, as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, Transforming Growth Factor alpha (TGF-alpha), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-alpha and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn, *supra*; Masui et al. *Cancer Research* 44:1002-1007 (1984); and Wu et al. *J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the ErbB family, p185neu, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science*, 235:177-182 (1987); Slamon et al., *Science*, 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder.

Antibodies directed against the rat p185neu and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185neu (see, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and WO94/22478). Drebin et al. (*Oncogene* 2:273-277 (1988)) report that mixtures of antibodies reactive with two distinct regions of p185neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice (see also U.S. Pat. No. 5,824,311).

Hudziak et al., (*Mol. Cell. Biol.* 9(3):1165-1172 (1989)) describe the generation of a panel of anti-HER2 antibodies, which were characterized using the human breast tumor cell line SKBR3. Relative cell proliferation of the SKBR3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5, which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to

US 8,440,402 B2

11

a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-alpha (see, also, U.S. Pat. No. 5,677,171). The anti-HER2 antibodies discussed in Hudziak et al. were further characterized (Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3): 59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sliwkowski et al. *J. Biol. Chem.* 269(20):14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997)).

A recombinant humanized IgG1 version of the murine anti-HER2 antibody 4D5 (rhuMAb HER2 or HERCEPTIN®; commercially available from Genentech, Inc., South San Francisco) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). HERCEPTIN® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. The current treatment protocol employs IHC to determine HER2 protein overexpression.

Other anti-HER2 antibodies with various properties have been described (Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., (*Proc. Natl. Acad. Sci. USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); PCT Publication No. WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; Klapper et al. *Oncogene* 14:2099-2109 (1997); and PCT Publication No. WO 98/17797).

Homology screening has resulted in the ErbB receptor family members: HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968; Kraus et al., *Proc. Natl. Acad. Sci. USA* 86:9193-9197 (1989)) and HER4 (European Patent Application No. EP 599 274; Plowman et al., *Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and Plowman et al., *Nature*, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The ErbB receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of ErbB ligands (Earp et al., *Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands: Epidermal Growth Factor (EGF), Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, Heparin Binding Epidermal Growth Factor (HB-EGF), betacellulin, and epiregulin (Groenen et al. *Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta, and gamma heregulins (Holmes et al., *Science*, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al., *Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors

12

(GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF) (for a review, see Groenen et al., *Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee et al. *Pharm. Rev.* 47:51-85 (1995)). Recently, two additional ErbB ligands were identified: neuregulin-2 (NRG-2), which is reported to bind either HER3 or HER4 (Chang et al., *Nature*: 387 509-512 (1997); and Carraway et al. *Nature* 387:512-516 (1997)) and neuregulin-3, which binds HER4 (Zhang et al., (*Proc. Natl. Acad. Sci. USA*) 94(18): 9562-7 (1997)). HB-EGF, betacellulin, and epiregulin also bind to HER4.

While EGF and TGF-alpha do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase, (Earp et al., supra.) Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., *J. Biol. Chem.*, 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al., *Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey et al., *Proc. Natl. Acad. Sci. USA* 92:1431-1435 (1995); and Lewis et al., *Cancer Res.*, 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8 (1994)).

Detecting Gene Amplification

The present invention contemplates using any technique to detect gene amplification. (see, Boxer, *J. Clin. Pathol.* 53: 19-21 (2000)). These techniques include in situ hybridization (Stoler, *Clin. Lab. Med.* 12:215-36 (1990), using radioisotope or fluorophore-labeled probes; polymerase chain reaction (PCR); quantitative Southern blotting, and other techniques for quantitating individual genes. Preferably probes or primers selected for gene amplification evaluation are highly specific, to avoid detecting closely related homologous genes.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. A hapten or epitope that is immunospecifically bound by an antibody can also serve as a label.

The term "fluorescently labeled nucleic acid probe" refers to a probe comprising (1) a nucleic acid having a sequence rendering it capable of hybridizing with a target nucleic acid sequence and (2) a fluorescent label. Preferably such hybridization is specific, i.e., it can occur under high stringency conditions.

Sample Preparation

Any tissue sample from a subject may be used. Examples of tissue samples that may be used include, but are not limited to, breast, prostate, ovary, colon, lung, endometrium, stomach, salivary gland or pancreas. The tissue sample can be obtained by a variety of procedures including, but not limited to surgical excision, aspiration, or biopsy. The tissue may be

US 8,440,402 B2

13

fresh or frozen. In one embodiment, the tissue sample is fixed and embedded in paraffin or the like.

The tissue sample may be fixed (i.e., preserved) by conventional methodology (See e.g., *Manual of Histological Staining Method of the Armed Forces Institute of Pathology*, 3rd Edition Lee G. Luna, H T (ASCP) Editor, The Blakston Division McGraw-Hill Book Company: New York; (1960); *The Armed Forces Institute of Pathology Advanced Laboratory Methods in Histology and Pathology* (1994) Ulreka V. Mikel, Editor, Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.). One of skill in the art will appreciate that the choice of a fixative is determined by the purpose for which the tissue is to be histologically stained or otherwise analyzed. One of skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin's or paraformaldehyde, may be used to fix a tissue sample.

Generally, the tissue sample is first fixed and is then dehydrated through an ascending series of alcohols, infiltrated, and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. By way of example, the tissue sample may be embedded and processed in paraffin by conventional methodology. Examples of paraffin that may be used include, but are not limited to, Paraplast, Broloid, and Tissuemay. Once the tissue sample is embedded, the sample may be sectioned by a microtome or the like. By way of example for this procedure, sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine, and the like. For example, the paraffin embedded sections may be attached to positively charged slides, slides coated with poly-L-lysine.

If paraffin has been used as the embedding material, the tissue sections are generally deparaffinized and rehydrated to water. The tissue sections may be deparaffinized by several conventional standard methodologies. For example, xylenes and a gradually descending series of alcohols may be used. Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Tex.) may be used.

Fluorescence In Situ Hybridization (FISH)

In situ hybridization is generally carried out on cells or tissue sections fixed to slides. In situ hybridization may be performed by several conventional methodologies (see, e.g., Leitch et al., *In Situ Hybridization: A Practical Guide*, Oxford BIOS Scientific Publishers, Microscopy Handbooks v. 27 (1994)). In one in situ procedure, fluorescent dyes (such as fluorescein isothiocyanate (FITC) which fluoresces green when excited by an Argon ion laser) are used to label a nucleic acid sequence probe that is complementary to a target nucleotide sequence in the cell. Each cell containing the target nucleotide sequence will bind the labeled probe producing a fluorescent signal upon exposure, of the cells to a light source of a wavelength appropriate for excitation of the specific fluorochrome used. A "target nucleotide sequence" is a sequence specific for an over-expressed tumor antigen, such as ErbB. FISH analysis can be used in conjunction with other assays, including without limitation morphological staining (of serial sections or the same section; see PCT Publication No. WO 00/20641, specifically incorporated herein by reference).

14

Various degrees of hybridization stringency can be employed. As the hybridization conditions become more stringent, a greater degree of complementarity is required between the probe and target to form and maintain a stable duplex. Stringency is increased by raising temperature, lowering salt concentration, or raising formamide concentration. Adding dextran sulfate or raising its concentration may also increase the effective concentration of labeled probe to increase the rate of hybridization and ultimate signal intensity. After hybridization, slides are washed in a solution generally containing reagents similar to those found in the hybridization solution with washing time varying from minutes to hours depending on required stringency. Longer or more stringent washes typically lower nonspecific background but run the risk of decreasing overall sensitivity.

Probes used in the FISH analysis may be either RNA or DNA oligonucleotides or polynucleotides and may contain not only naturally occurring nucleotides but their analogs like digoxigenin dCTP, biotin dCTP 7-azaguanosine, azidothymidine, inosine, or uridine. Other useful probes include peptide probes and analogues thereof, branched gene DNA, peptidomimetics, peptide nucleic acid (PNA), and/or antibodies.

Probes should have sufficient complementarity to the target nucleic acid sequence of interest so that stable and specific binding occurs between the target nucleic acid sequence and the probe. The degree of homology required for stable hybridization varies with the stringency of the hybridization medium and/or wash medium. Preferably, completely homologous probes are employed in the present invention, but persons of skill in the art will readily appreciate that probes exhibiting lesser but sufficient homology can be used in the present invention (see e.g., Sambrook, J., et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Press, (1989)).

One of skill in the art will appreciate that the choice of probe depends on the characteristics of the target gene of interest. Examples of amplification include, but are not limited to, her2/neu in breast and ovarian cancer, n-myc in neuroblastoma, c-myc in small cell lung cancer. By way of example for evaluating her2/neu amplification a probe spanning a 140 kb region on the long arm of chromosome 17 containing the her2/neu gene (17q11.2-17q12) may be used. A probe for the -satellite sequences in the centromeric region of chromosome 17(D17Z1) may be used to evaluate for aneuploidy of chromosome 17 as a source or cause for her2/neu amplification. For example, a cocktail version of these probes may be obtained from Vysis, Inc. where each probe is directly labeled with easily distinguishable fluorophores, such as SPECTRUM ORANGE® and SPECTRUM GREEN®.

Probes may also be generated and chosen by several means including, but not limited to, mapping by in situ hybridization, somatic cell hybrid panels, or spot blots of sorted chromosomes; chromosomal linkage analysis; or cloned and isolated from sorted chromosome libraries from human cell lines or somatic cell hybrids with human chromosomes, radiation somatic cell hybrids, microdissection of a chromosome region, or from yeast artificial chromosomes (YACs) identified by PCR primers specific for a unique chromosome locus or other suitable means like an adjacent YAC clone. Probes may be genomic DNA, cDNA, or RNA cloned in a plasmid, phage, cosmid, YAC, Bacterial Artificial Chromosomes (BACs), viral vector, or any other suitable vector. Probes may be cloned or synthesized chemically by conventional methods. When cloned, the isolated probe nucleic acid fragments are typically inserted into a vector, such as lambda phage,

US 8,440,402 B2

15

pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host (see, e.g., Sambrook, supra).

Probes are preferably labeled with a fluorophor. Examples of fluorophores include, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocrytherin, phycocyanin, or commercially available fluorophors such as SPECTRUM ORANGE® and SPECTRUM GREEN®, and/or derivatives of any one or more of the above. Multiple probes used in the assay may be labeled with more than one distinguishable fluorescent or pigment color. These color differences provide a means to identify the hybridization positions of specific probes. Moreover, probes that are not separated spatially can be identified by a different color light or pigment resulting from mixing two other colors (e.g., light red+green=yellow), pigment (e.g., blue+yellow=green), or by using a filter set that passes only one color at a time.

Probes can be labeled directly or indirectly with the fluorophor, utilizing conventional methodology. Additional probes and colors may be added to refine and extend this general procedure to include more genetic abnormalities or serve as internal controls. By way of example the her2/neu gene is in chromosome 17, and as an internal control a probe for satellite sequences specific for chromosome 17 (D17Z1) may be used (Vysis, Inc.) to prove diploidy in areas of non-malignant cells and/or to establish the presence or absence of chromosome 17 aneusomy in areas of her2/neu amplification.

After processing for FISH, the slides may be analyzed by standard techniques of fluorescence microscopy (see, e.g., Ploem and Tanke, Introduction to Fluorescence Microscopy, Oxford University Press: New York (1987)). Briefly, each slide is observed using a microscope equipped with appropriate excitation filters, dichromic, and barrier filters. Filters are chosen based on the excitation and emission spectra of the fluorochromes used. Photographs of the slides may be taken with the length of time of film exposure depending on the fluorescent label used, the signal intensity and the filter chosen. For FISH analysis the physical loci of the cells of interest determined in the morphological analysis are recalled and visually conformed as being the appropriate area for FISH quantification.

In order to correlate IHC with FISH, one may use a computer-driven, motorized stage which stores location of coordinates. This may be used to evaluate the same area by two different analytical techniques. For example, color images of the morphologically stained areas may be captured and saved using a computer-assisted cooled CCD camera. The same section may be subsequently taken through the FISH procedure, the stored locations recalled, and the designated areas scored for the presence of fluorescent nuclear signals. A similar procedure for IHC followed by FISH is contemplated.

Typically, hundreds of cells are scanned in a tissue sample and quantification of the specific target nucleic acid sequence is determined in the form of fluorescent spots, which are counted relative to the number of cells. Deviation of the number of spots in a cell from a norm (e.g., such as probing for the her2/neu gene in a normal cell will produce two copies, abnormal greater than two) is indicative of a greater likelihood of benefit from a tumor antigen-specific antibody therapy, e.g., an ErbB antagonist therapy. As exemplified infra, her2 gene amplification provides a much more effective indication of the likelihood that an anti-HER2 antibody therapy will be effective.

Pharmaceutical Formulations

Therapeutic formulations of the antagonists, e.g., antibodies, used in accordance with the present invention are pre-

16

pared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICTM or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2, ErbB3, ErbB4, vascular endothelial factor (VEGF), or an antibody that binds to a different epitope on the target ErbB, in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, a chemotherapeutic, a cytokine, growth inhibitory agent and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 17th edition, Osol, A. Ed.

The formulations to be used for in vivo administration are preferably, and in the case of humans, must be, sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over

US 8,440,402 B2

17

100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Treatment with the Anti-ErbB Antagonists

It is contemplated that, according to the present invention, the anti-ErbB antibodies or other antagonists may be used to treat various conditions characterized by overexpression and/or activation of the ErbB receptor in patients who have been found to have an amplified erbB gene. Exemplary conditions or disorders include benign or malignant tumors (e.g. renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytic, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelec, inflammatory, angiogenic and immunologic disorders.

The antibodies, chemotherapeutic agents and any other active agents of the invention are administered to a human patient in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB antibody and a chemotherapeutic agent, e.g., a taxoid. The present invention contemplates administration of cocktails of different chemotherapeutic agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616 812) in dosages known for such molecules.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells (tumor resection) and/or radiation therapy.

For the prevention or treatment of disease, the appropriate dosage of antagonist, e.g., antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the

18

discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Pharmaceutical Packages

Articles of Manufacture

In a related aspect of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, optionally labeled, and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials, such as glass or plastic. The container holds a composition that is effective for treating the condition and preferably has a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-tumor antigen therapeutic antibody or an ErbB antagonist, e.g., an anti-ErbB antibody. A label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. This second buffer can be used to reconstitute the active agent, if that is provided as a lyophilysate or dried powder, or to dilute a concentrated preparation of the active agent. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In addition, the article of manufacture comprises a package insert or inserts with instructions for use in patients who have been found to have erbB gene amplification, e.g., by FISH testing. Such patients may be subjects who, by IHC, would be excluded from treatment with the ErbB antagonist, e.g., patients who score a 0 or 1+ using an anti-HER2 antibody.

Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990

US 8,440,402 B2

19

Further details of the invention are illustrated by the following non-limiting Examples.

Example 1

Concordance Between the Clinical Trials Assay (CTA) and Fluorescence In Situ Hybridization (FISH) in the HERCEPTIN® Pivotal Trials

Overexpression of HER2 at the 2+ or 3+ level by immunohistochemistry (IHC) was required for enrollment in the pivotal HERCEPTIN® metastatic breast cancer trials. The Clinical Testing Assay (CTA) involves two separate IHC assays performed with either monoclonal antibodies 4D5 (after protease digestion of the formalin fixed sample) or CB11 (after heat treatment of the formalin fixed sample). Subjects were eligible if either assay was scored at 2+ or 3+. If both were performed, the final score was the higher of the two results.

Concordance between the CTA and another IHC, HERCEPTIN® (HT), is 79%. This was the basis for FDA approval of HT to aid in the selection of patients for HERCEPTIN® therapy.

This Example describes a similar concordance study, utilizing clinical material submitted for screening for the HERCEPTIN® pivotal trials, that compares the CTA to her2/neu gene amplification measured by the PathVysion FISH assay. In the pivotal trials, 5998 subjects were screened for HER2 expression; 1915 (32%) were positive by the CTA and 4083 (68%) were negative. A random sample of 623 specimens (1:1 ratio of positive:negative) were selected for this analysis, 317 CTA+ and 306 CTA-. Specimens were not freshly cut from blocks. They had been stored between 2 and 4 years as 4-6µ sections on glass slides. Each section was assayed for her2/neu amplification using the protocol specified in the package insert of the PathVysion assay. Amplification was defined as a signal ratio of greater than or equal to 2. The results are shown in Table 1.

TABLE 1

FISH/CTA Concordance						
		CTA				
		0	1+	2+	3+	
FISH	-	207	28	67	21	
	+	7	2	21	176	
		4%	7%	24%	89%	529

FISH+ = HER2:CEP17 signal ratio ≥ 2
Concordance = 82% (79-85%)

For the total 623 specimens tested, a FISH signal result was obtained in 529. Assay failure occurred in 19.9% of CTA- and 10.4% CTA+ samples. Amplification in the 0, 1+, 2, and 3+ groups was 4.2%, 6.7%, 23.9%, and 89.3%, respectively. The sample concordance was 81.3%, similar to the CTA/HT concordance of 79%. Single copy overexpression was 31%, predominantly in the 2+ group. Amplification was rarely (4.6%) noted in the CTA- group. The higher assay failure rate in the CTA- group may be due to non-assay related factors such as tissue fixation. These may have also resulted in false negative results for IHC.

These data were closely interpreted to suggest that her2/neu amplification status may have unexpectedly superior predictive value for identifying patients who are more likely to benefit from HERCEPTIN® treatment as compared to HERCEPTIN®. The observation that only 24% of 2+ patients

20

are FISH+ suggest that this sub-group may have less predictable treatment outcomes when selected by IHC only. Identification of FISH+ patients in the 1+ and 0 sub-groups might identify subjects who, though failing the IHC criteria for HERCEPTIN® treatment, would likely benefit from HERCEPTIN® treatment. A direct analysis of HERCEPTIN® benefit based on FISH score compared to IHC score is presented in Example 2.

Example 2

FISH/Clinical Outcome Study

This example links the results from three HERCEPTIN® Trials with FISH status. In this study, 805 subjects were selected at random from all three trials. Of these, 167 lacked slides. Another 78 assays (9.7%) failed. Thus, formalin-fixed cut sections stored between 2.5 and 4.5 years from 540 subjects provided the sample pool for this study. There were no imbalances in demographics or prognostic indicators in these samples. Results are reported for different treatment groups.

Correlation of FISH status with response was evaluated for patients who received HERCEPTIN® as a second or third line therapy. These data are reported for 2+ and 3+ (by CTA) subjects in Table 2.

TABLE 2

FISH/Response with single agent HERCEPTIN®, 2nd or 3rd line therapy, 2+/3+ Combined		
	FISH+	FISH-
Response	21	0
No response	84	37
response rate	20% (12.5-27.5%)	0% (0.7%)

N = 142

The 20% response rate of FISH+ subjects unexpectedly exceeds the 15% response rate of 2+ and 3+ patients in this study and 14% response rate observed in patients selected by CTA with a 2+ or 3+ immunohistochemistry score during the pivotal trials. Thus, while FISH correlates well with NC to about the same degree as another NC assay, the Hercep Test, as shown in Example 1, it unexpectedly is superior in identifying patients who are more likely to benefit from HERCEPTIN® therapy.

When these data were broken down into the components 3+ and 2+ subjects, the same 20% response rate of FISH+ subjects was seen (Tables 3 and 4).

TABLE 3

FISH/Response with single agent HERCEPTIN®, 2nd or 3rd line therapy, 3+ subgroup		
	FISH+	FISH-
Response	18	0
No response	72	17
response rate	20% (12-28%)	0% (0-14%)

N = 107

US 8,440,402 B2

21

TABLE 4

FISH/Response with single agent HERCEPTIN®, 2nd or 3rd line therapy, 2+ subgroup		
	FISH+	FISH-
Response	3	0
No response	12	20
response rate	20%	0%
	(1-40%)	(0-14%)

N = 35

In the 3+ sub-group, the FISH+ response rate (20%) was very close but still exceeded the 17% response rate of 3+ subjects. The 2+ subgroup showed a much greater difference, with only a 9% response rate versus 20% by FISH+ selection. These data show that FISH+ status (her2 gene amplification) greatly increases the likelihood of response to HERCEPTIN®.

Data were also evaluated for patient responses to HERCEPTIN® as a first line therapy (Table 5).

TABLE 5

FISH/Response with single agent HERCEPTIN® as 1st line therapy, 2+/3+ combined		
	FISH+	FISH-
Response	17	1
No response	24	20
response rate	41%	20%
	(26-56%)	(0-14%)

N = 62

The 41% response rate of FISH+ subjects was notably greater than the 27% response rate of 3+, 2+ subjects.

The surprising increase in likelihood of beneficial response based on FISH analysis extended to responses to chemotherapy plus HERCEPTIN®, as shown in Table 6. FISH+ subjects showed a much greater response to chemotherapy and HERCEPTIN® (54%) than FISH- (41%). Tables 7-9 contain more extensive data, broken down by different chemotherapeutic agents (adriamycin and cyclophosphamide, AC; and Paclitaxel, P) and different endpoints (response rate, time to progression, and survival) for HERCEPTIN® in combination with chemotherapy.

TABLE 6

FISH/Response rate to chemotherapy +/- HERCEPTIN®, 1st line therapy; 2+/3+ combined		
	C alone	C + H
FISH-	39%	41%
	(26-52%)	(27-55%)
FISH+	27%	54%
	(19-35%)	(45-63%)

N = 336

TABLE 7

Response rate of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	56*	42	41*	17	50*	32
3+	349	60*	42	49*	17	56*	31
FISH+	240	58*	40	49*	14	54*	27

*p < 0.05

22

TABLE 8

Time to progression (months) of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	7.8*	6.1	6.9*	2.7	7.4*	4.6
3+	349	8.1*	6.0	7.1*	3.0	7.8*	4.6
FISH+	240	7.8*	6.2	7.0*	3.2	7.3*	4.6

*p < 0.05

TABLE 9

Survival (months) of newly defined populations							
		H + Ac (n = 143)	AC (n = 138)	H + P (n = 92)	P (n = 96)	H + CT (n = 235)	CT (n = 234)
2+/3+	469	27	21	22	18	25*	20
3+	349	31*	21	25	18	29*	20
FISH+	240	29*	20	25*	14	27*	18

*p < 0.05

These data uniformly confirm that FISH+ analysis, though correlating closely to IHC, provides a much more accurate indicator of likelihood of success with HERCEPTIN® treatment. Across the board, FISH+ selection has about 1/3 (30%) greater response rate than 2+/3+ IHC-selection. When focused on 2+ patients, FISH status provides a much more effective tool for patient selection. FISH status also identifies patients who, because of 0 or 1+ status as determined by IHC, would otherwise be excluded from treatment.

These observations have broad implications for ErbB receptor antagonist-based cancer therapies and anti-tumor antigen cancer therapies in general. Thus erbB antagonists, e.g., anti-erbB receptor antibodies like HERCEPTIN®, can have an increased likelihood of efficacy when administered to patients who are positive for erbB gene amplification, e.g., by a FISH test. This is certainly the case, based on these data, with HERCEPTIN®.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed:

1. A method for identifying and treating a patient disposed to respond favorably to a HER2 antibody, huMAb4D5-8, for treating breast cancer, which method comprises detecting her2 gene amplification in tumor cells in a tissue sample from the patient and treating the patient with her2 gene amplification with the HER2 antibody in an amount effective to treat the breast cancer, wherein the patient's tumor cells express HER2 at a 0 or 1+ level by immunohistochemistry.

2. The method of claim 1 wherein her2 gene amplification is detected by detecting fluorescence of a fluorescent-labeled nucleic acid probe hybridized to the gene.

US 8,440,402 B2

23

24

3. The method of claim 1 wherein a formaldehyde-fixed sample of the patient's tumor cells has been subjected to an immunohistochemistry assay and found to express HER2 at a 0 or 1+ level.

4. The method of claim 3 wherein the immunohistochem- 5
istry assay is performed prior to detecting her2 gene amplifi-
cation.

5. The method of claim 1 which further comprises admin-
istering a cancer treating dose of a chemotherapeutic drug.

6. The method of claim 5 wherein the chemotherapeutic 10
drug is a taxoid.

* * * * *

EXHIBIT I



US008574869B2

(12) **United States Patent**
Kao et al.

(10) **Patent No.:** **US 8,574,869 B2**
(45) **Date of Patent:** **Nov. 5, 2013**

(54) **PREVENTION OF DISULFIDE BOND
REDUCTION DURING RECOMBINANT
PRODUCTION OF POLYPEPTIDES**

(75) Inventors: **Yung-Hsiang Kao**, San Mateo, CA
(US); **Michael W. Laird**, San Ramon,
CA (US); **Melody Trexler Schmidt**, San
Carlos, CA (US); **Rita L. Wong**,
Redwood City, CA (US); **Daniel P.
Hewitt**, Sunnyvale, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco,
CA (US)

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/354,223**

(22) Filed: **Jan. 19, 2012**

(65) **Prior Publication Data**

US 2013/0017598 A1 Jan. 17, 2013

Related U.S. Application Data

(63) Continuation of application No. 12/217,745, filed on
Jul. 8, 2008, now abandoned.

(60) Provisional application No. 60/948,677, filed on Jul. 9,
2007.

(51) **Int. Cl.**
C12P 1/00 (2006.01)
C12N 5/02 (2006.01)

(52) **U.S. Cl.**
USPC **435/41; 435/325**

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — Suzanne M Noakes

Assistant Examiner — Jae W Lee

(74) *Attorney, Agent, or Firm* — Morrison & Foerster LLP

(57)

ABSTRACT

Provided herein are methods for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

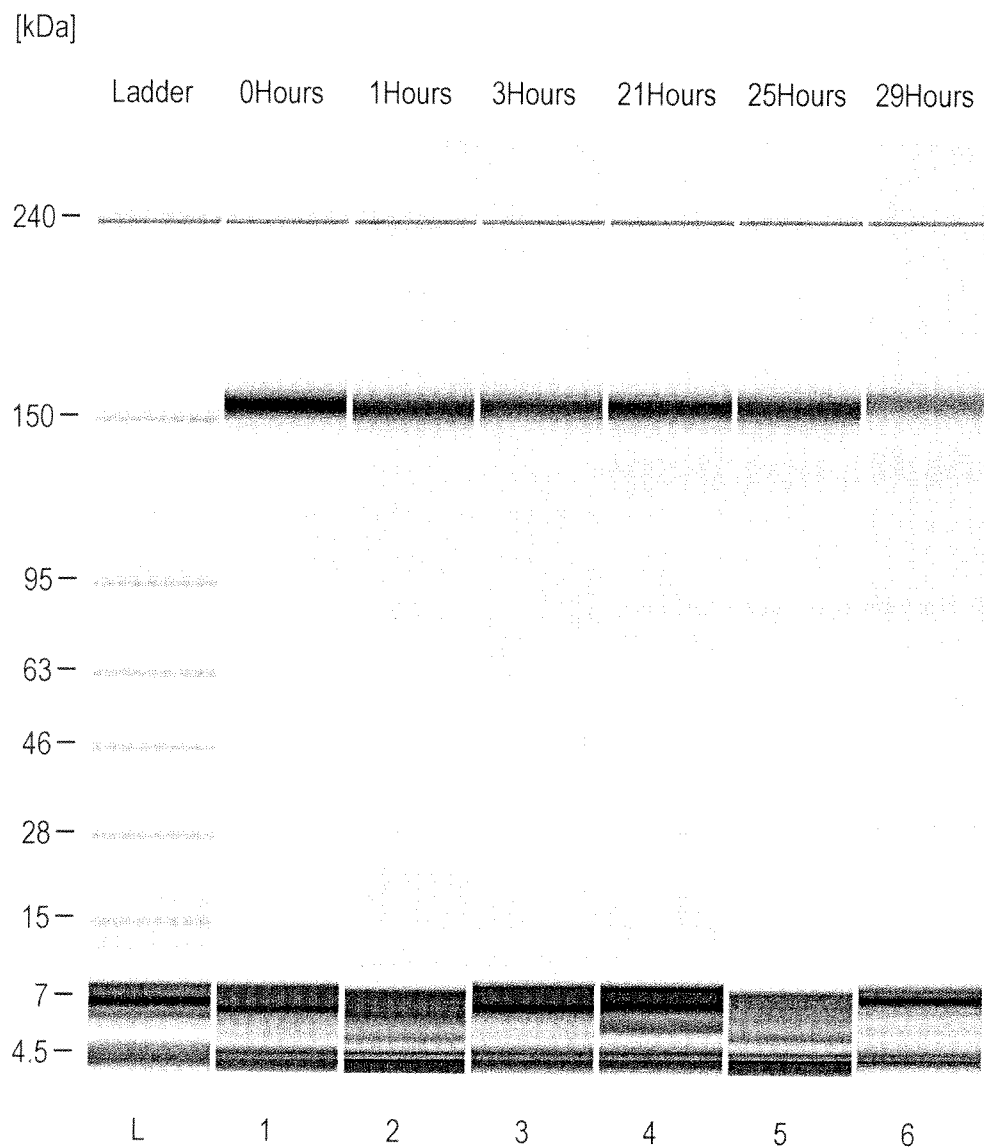
10 Claims, 40 Drawing Sheets

U.S. Patent

Nov. 5, 2013

Sheet 1 of 40

US 8,574,869 B2



Dialysis Experiment

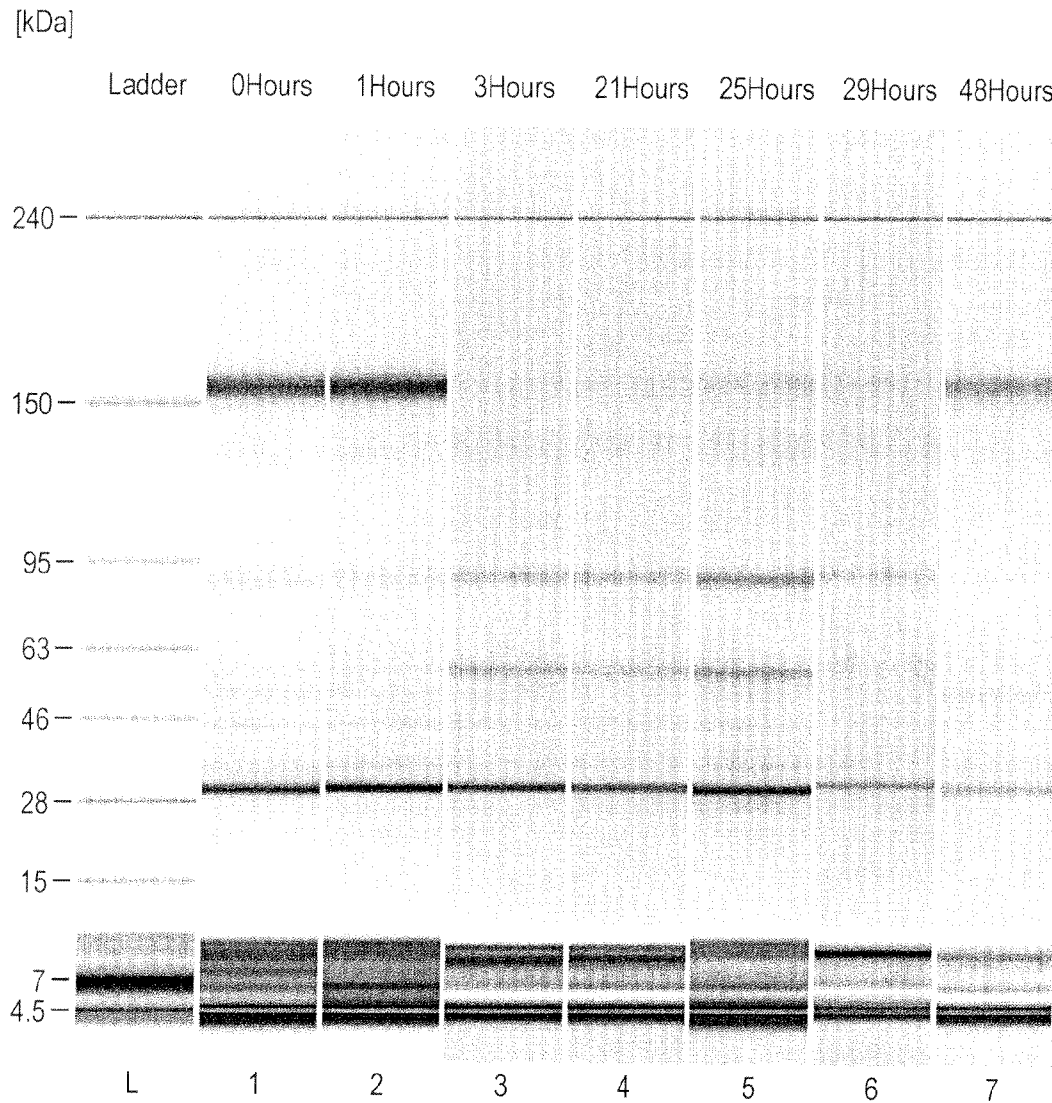
FIG. 1

U.S. Patent

Nov. 5, 2013

Sheet 2 of 40

US 8,574,869 B2



Dialysis Experiment

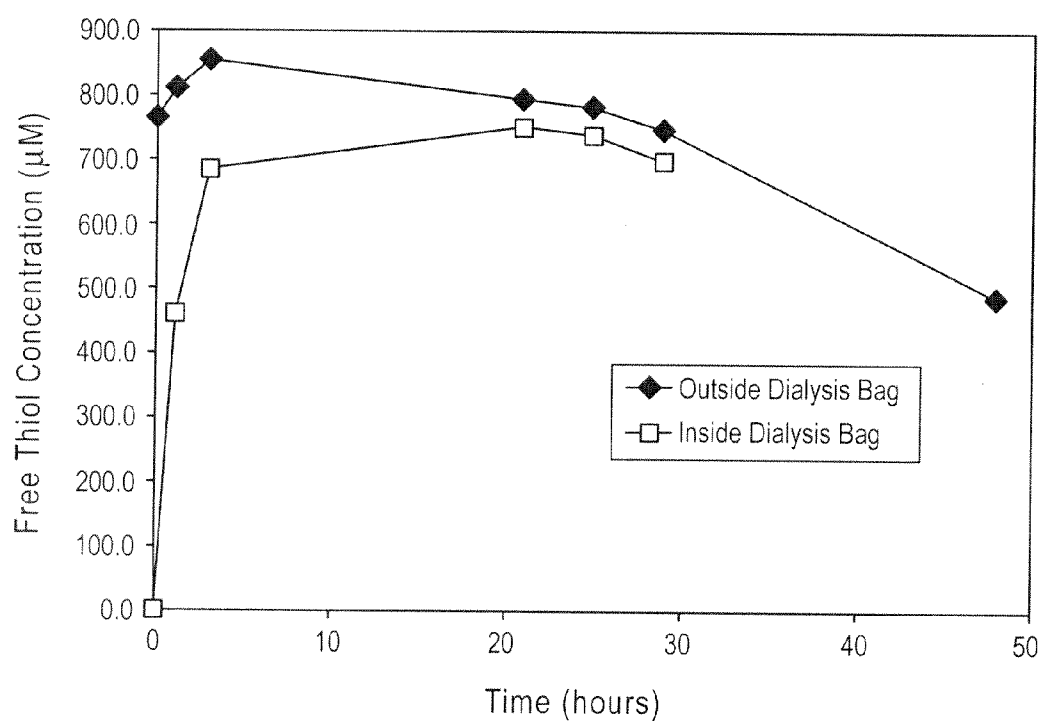
FIG. 2

U.S. Patent

Nov. 5, 2013

Sheet 3 of 40

US 8,574,869 B2



Free Thiol Levels from Dialysis Experiment

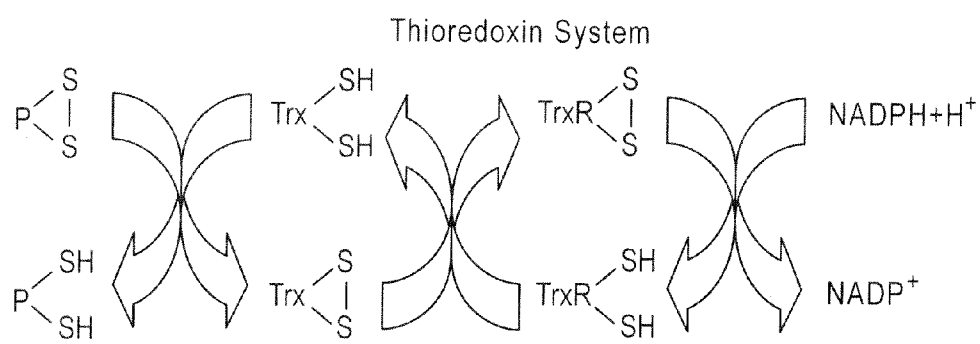
FIG. 3

U.S. Patent

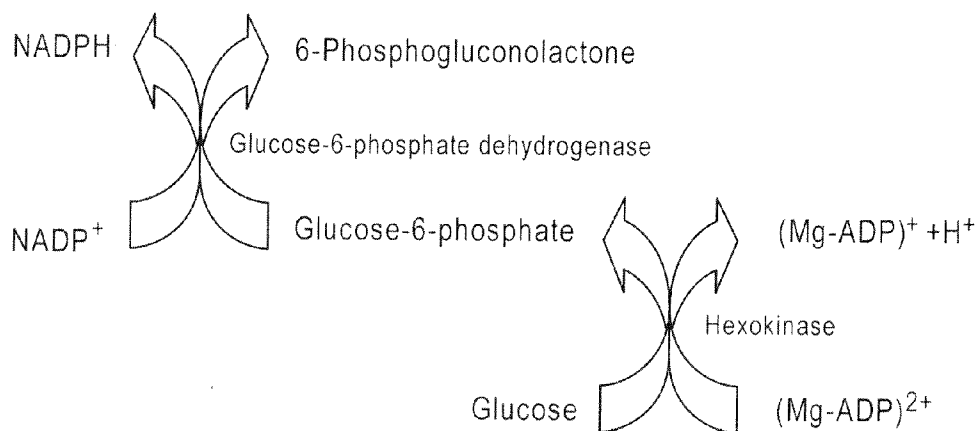
Nov. 5, 2013

Sheet 4 of 40

US 8,574,869 B2



First Reaction in Pentose Phosphate Pathway



First Reaction in Glycolysis

Thioredoxin System and Other Reactions Involved in Antibody Reduction

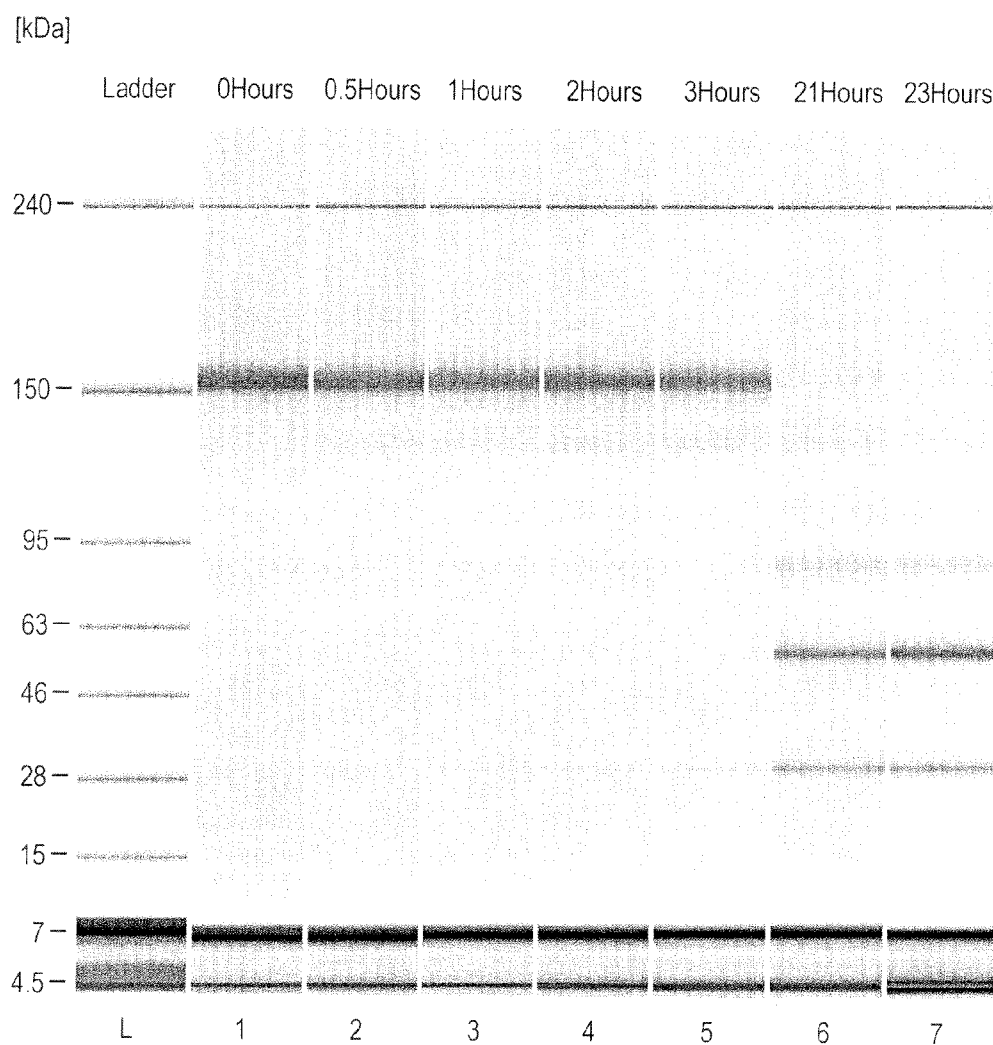
FIG. 4

U.S. Patent

Nov. 5, 2013

Sheet 5 of 40

US 8,574,869 B2



In Vitro Activity of Thioredoxin System

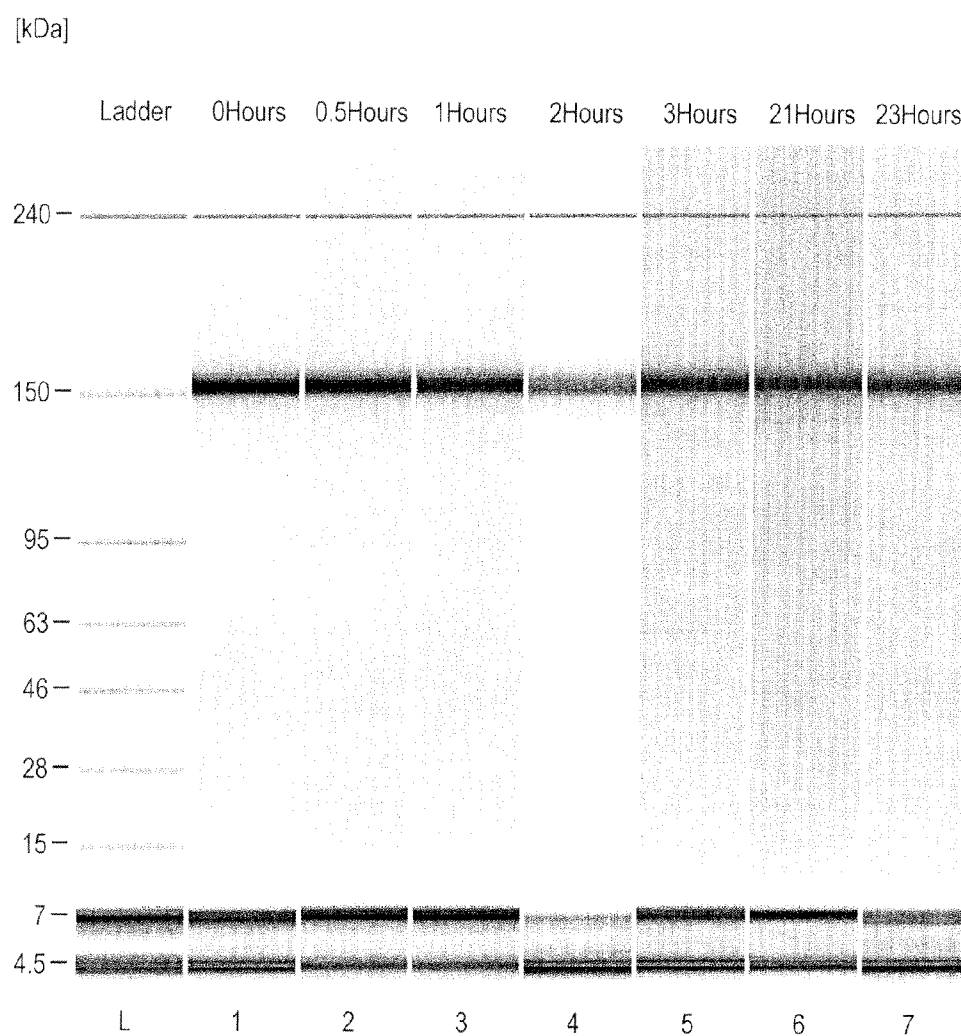
FIG. 5

U.S. Patent

Nov. 5, 2013

Sheet 6 of 40

US 8,574,869 B2



In vitro Activity of Thioredoxin System Inhibited by Aurothioglucose

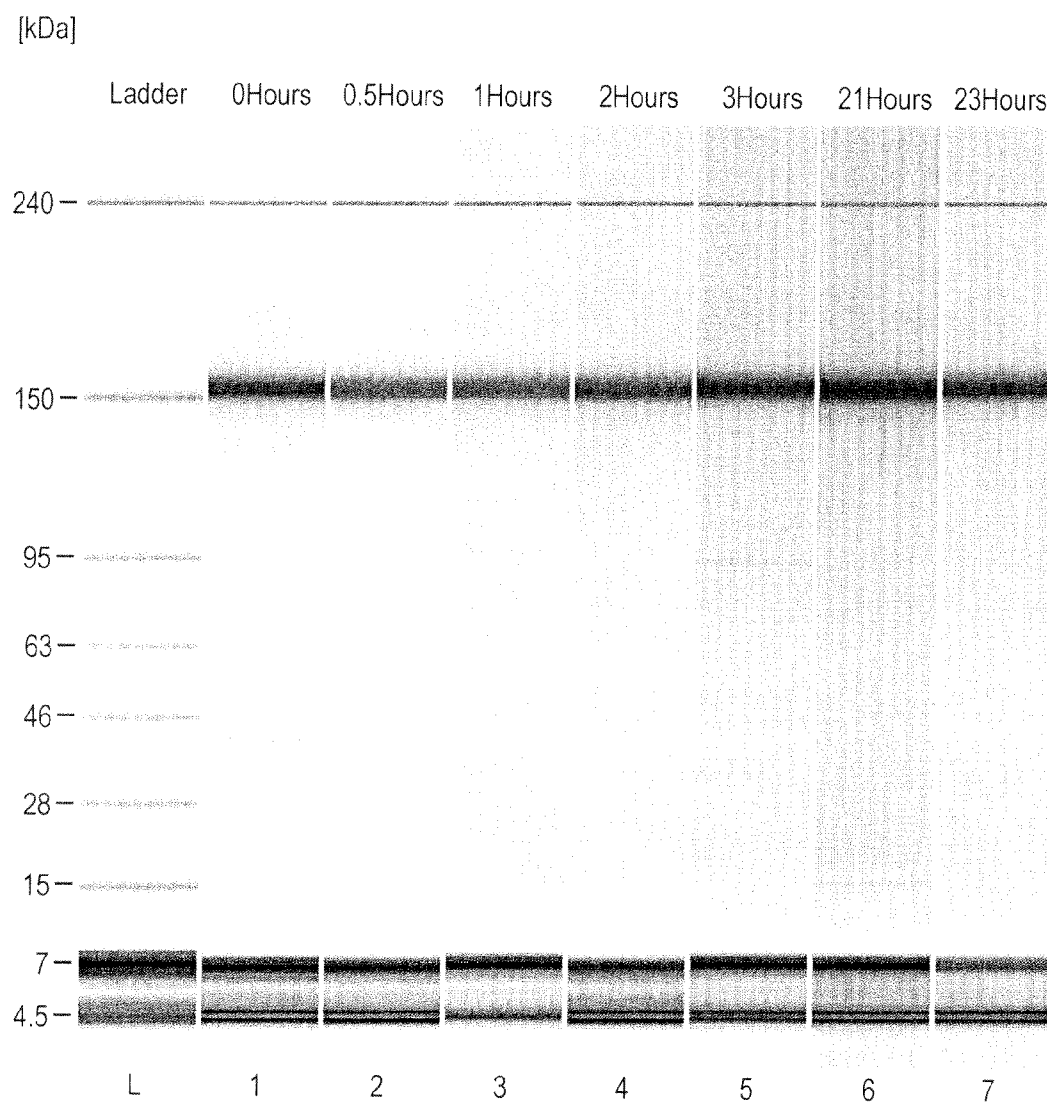
FIG. 6

U.S. Patent

Nov. 5, 2013

Sheet 7 of 40

US 8,574,869 B2



In vitro Activity of Thioredoxin System Inhibited by Aurothiomalate

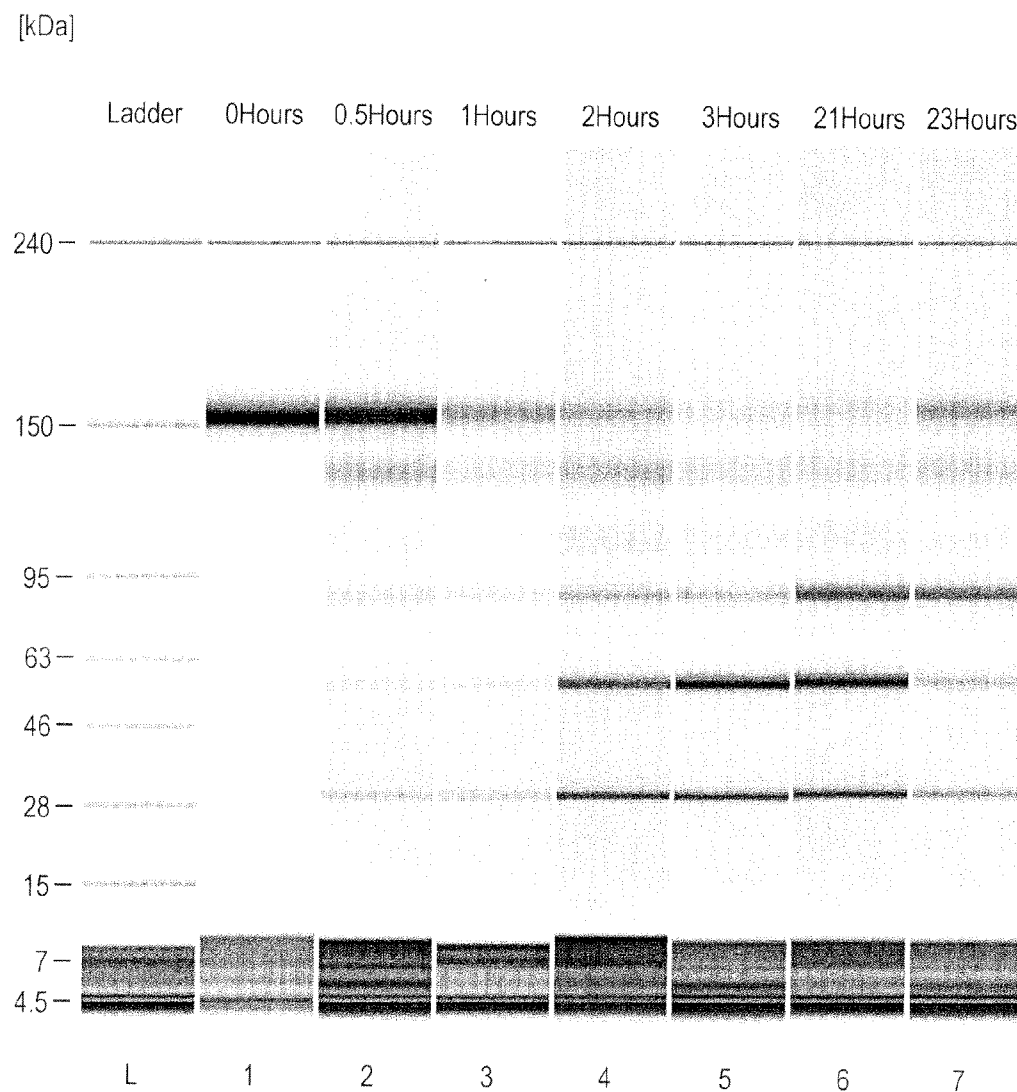
FIG. 7

U.S. Patent

Nov. 5, 2013

Sheet 8 of 40

US 8,574,869 B2



In vitro Activity of Thioredoxin System

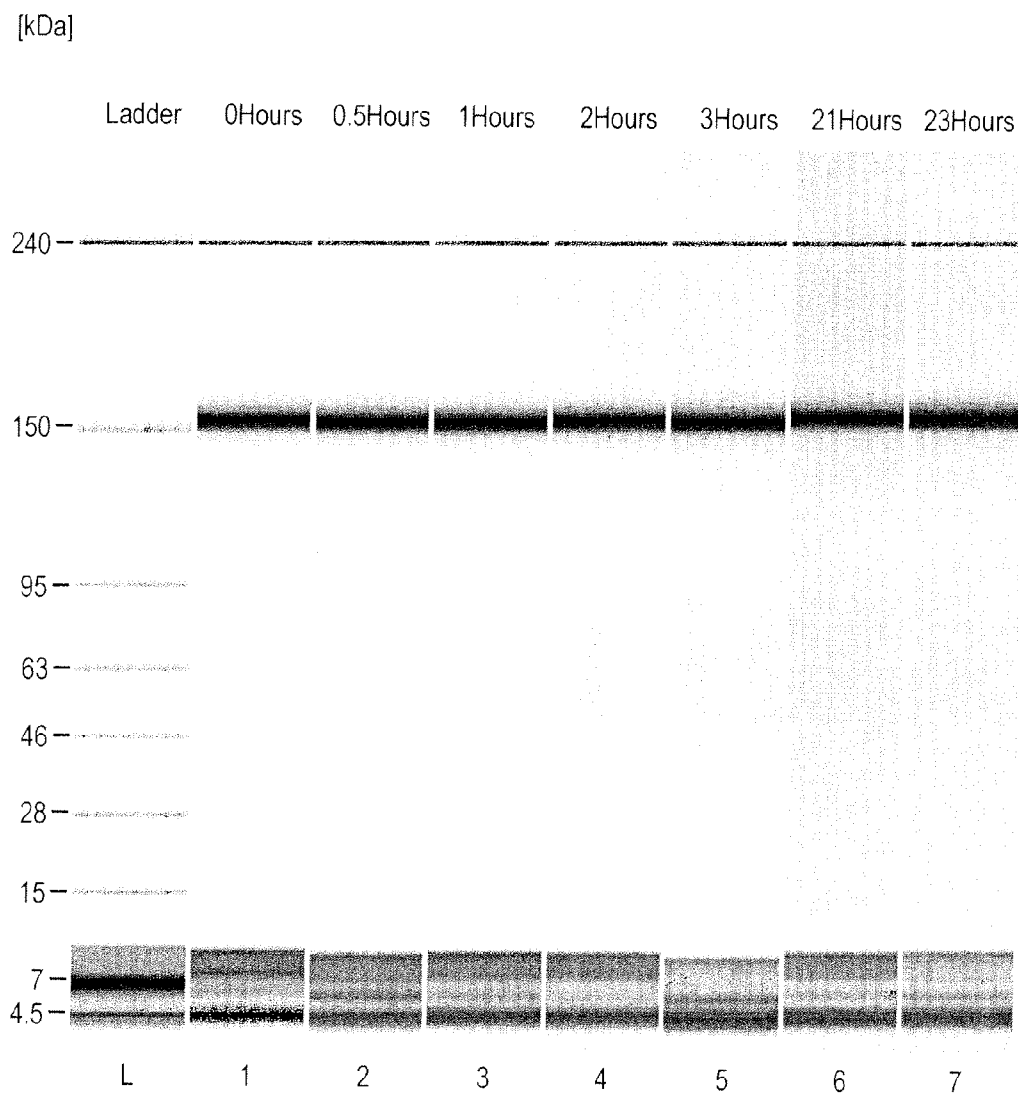
FIG. 8

U.S. Patent

Nov. 5, 2013

Sheet 9 of 40

US 8,574,869 B2



In vitro Activity of Thioredoxin System Inhibited by CuSO_4

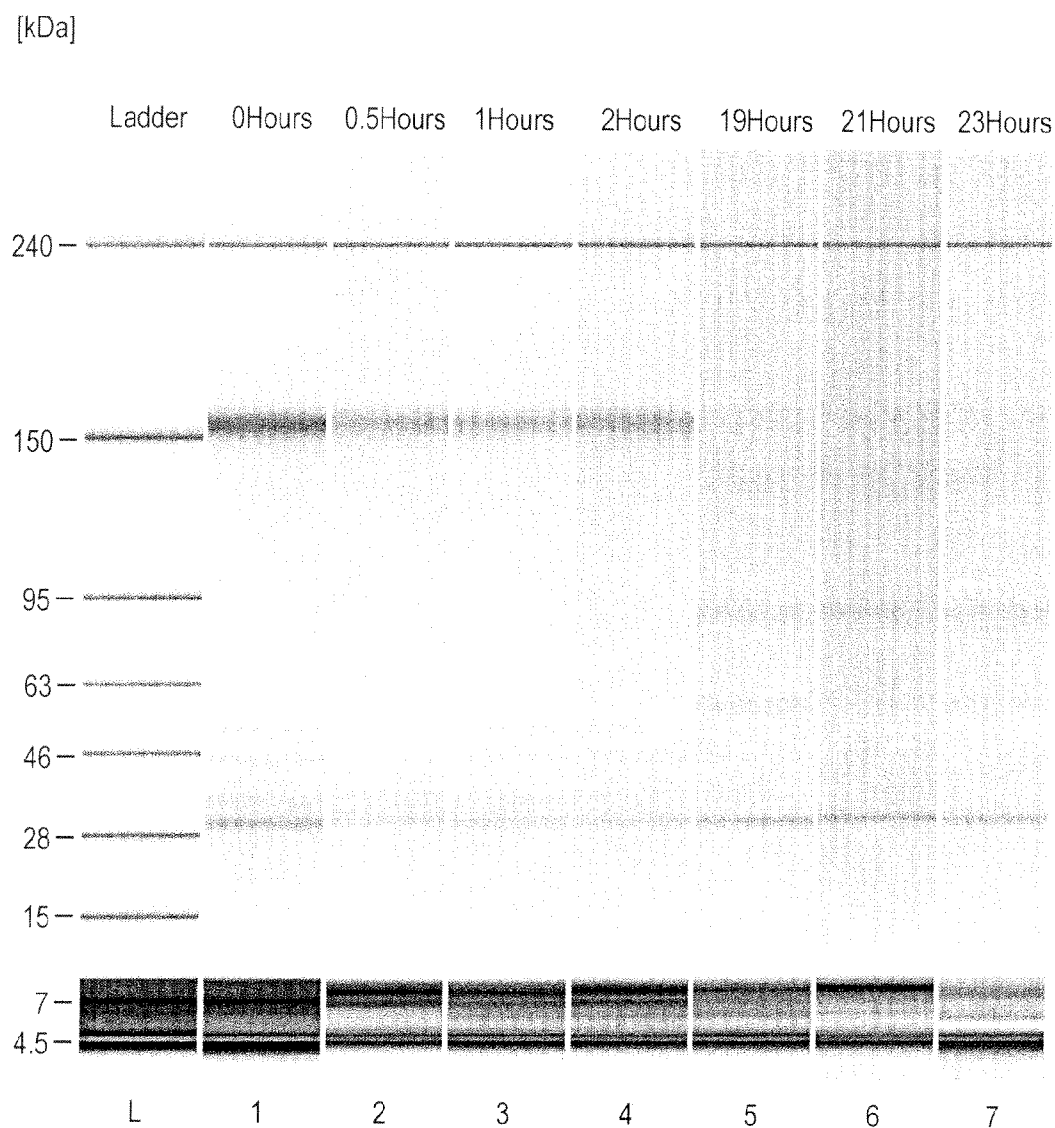
FIG. 9

U.S. Patent

Nov. 5, 2013

Sheet 10 of 40

US 8,574,869 B2



Ocrelizumab Reduction

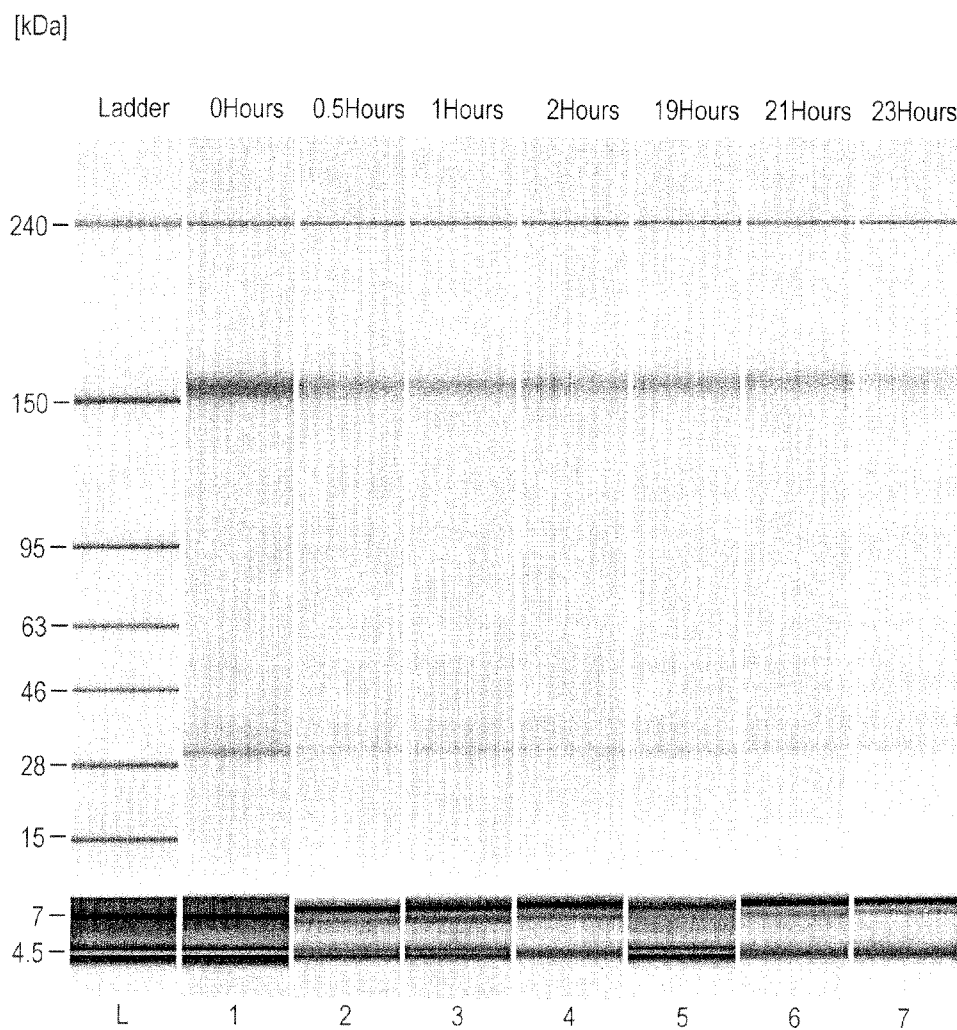
FIG. 10

U.S. Patent

Nov. 5, 2013

Sheet 11 of 40

US 8,574,869 B2



Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose

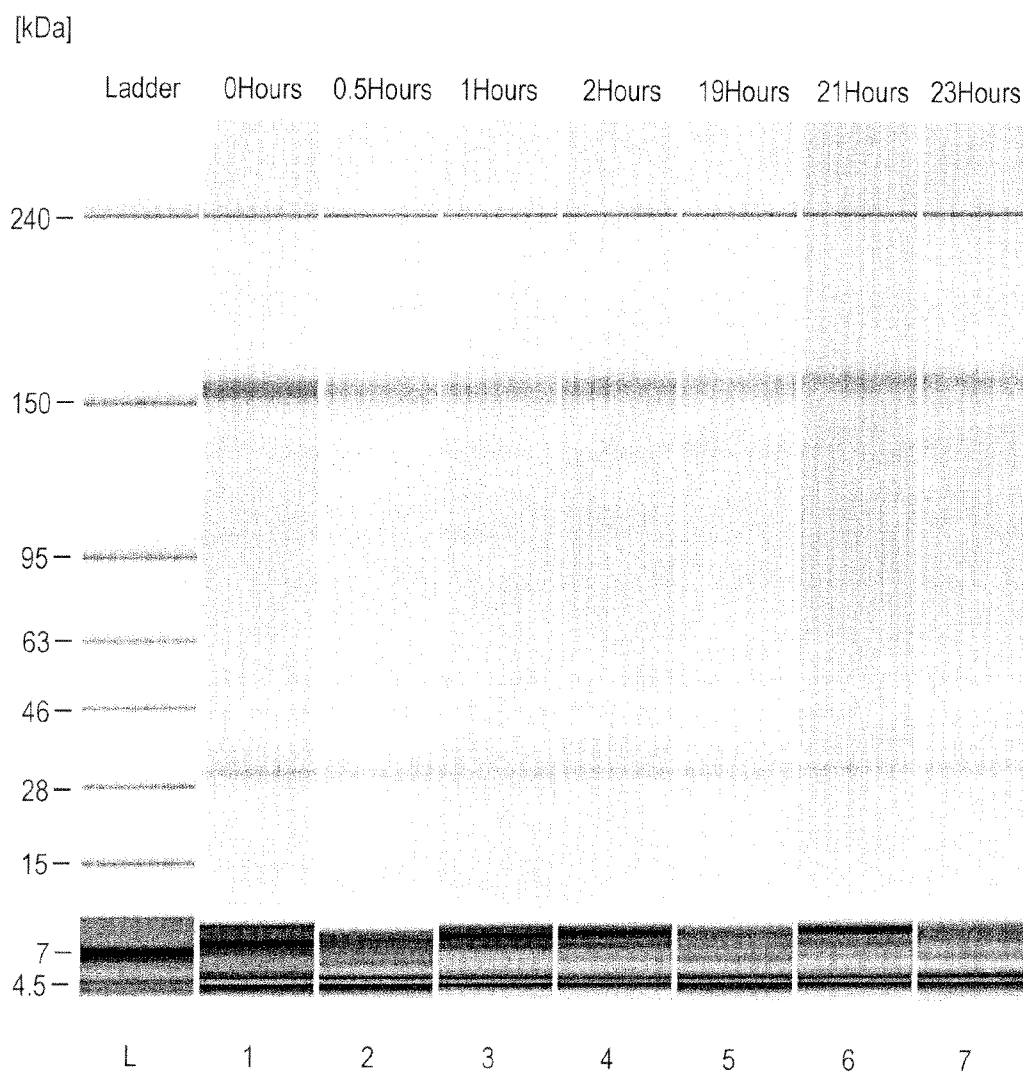
FIG. 11

U.S. Patent

Nov. 5, 2013

Sheet 12 of 40

US 8,574,869 B2



Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate

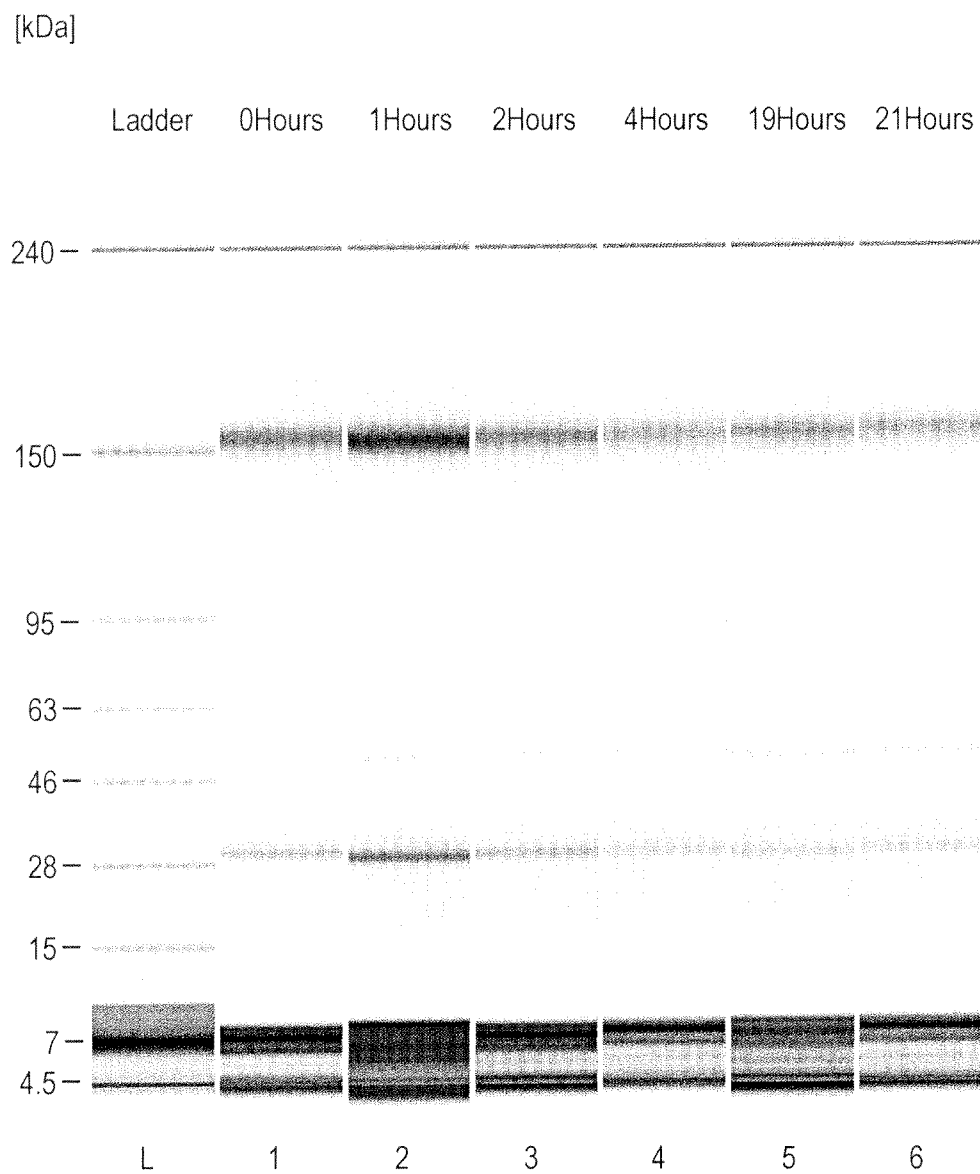
FIG. 12

U.S. Patent

Nov. 5, 2013

Sheet 13 of 40

US 8,574,869 B2



Losing Reduction Activity in HCCF

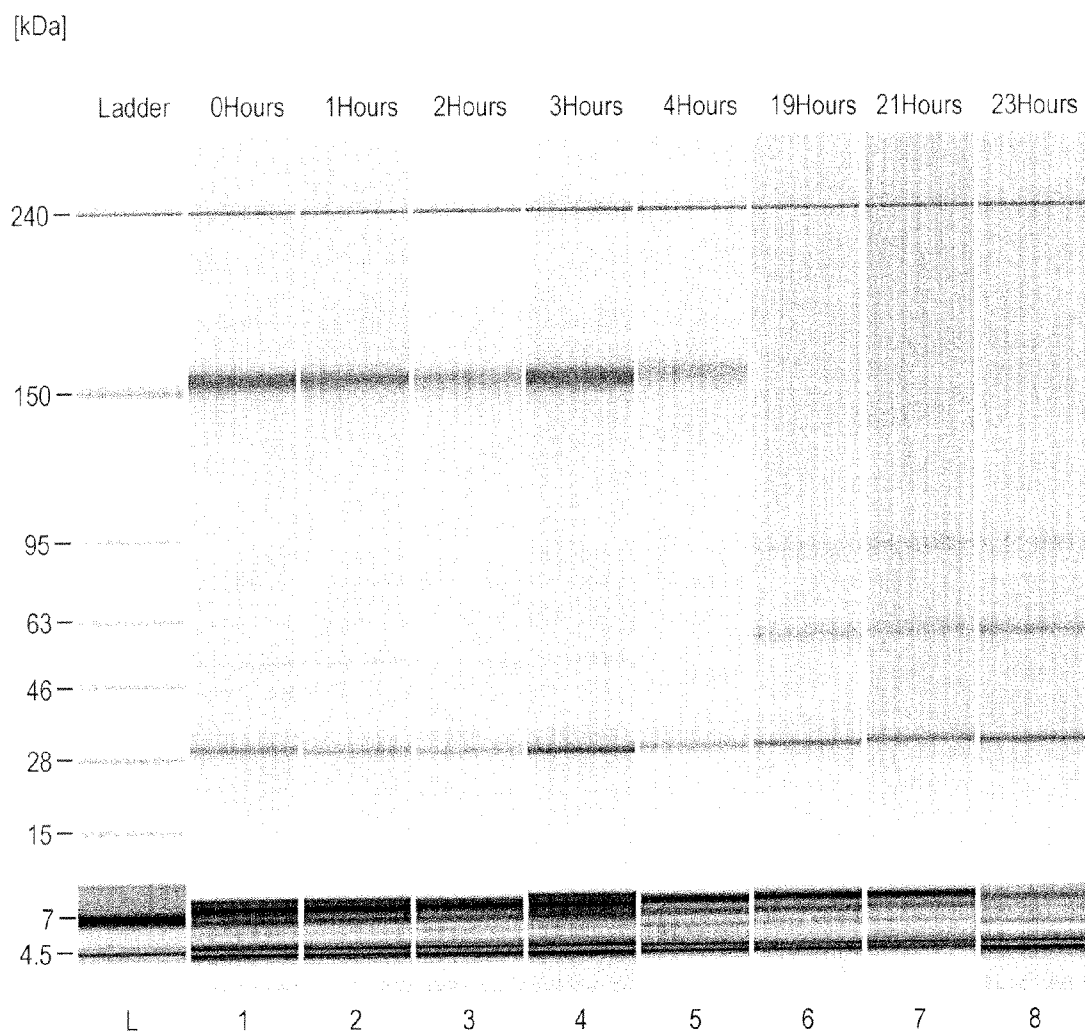
FIG. 13

U.S. Patent

Nov. 5, 2013

Sheet 14 of 40

US 8,574,869 B2



The Lost Reduction Activity in HCCF Restored by Addition of NADPH

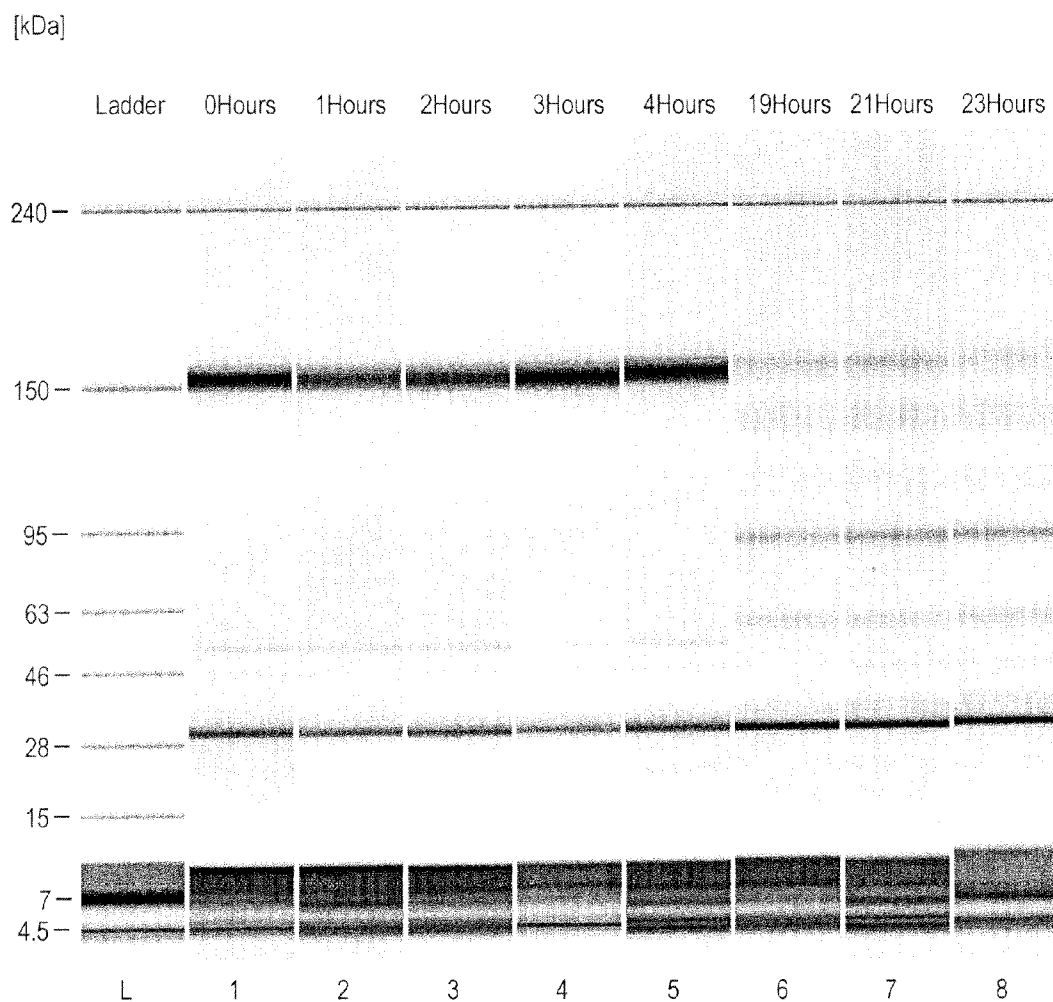
FIG. 14

U.S. Patent

Nov. 5, 2013

Sheet 15 of 40

US 8,574,869 B2



The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate

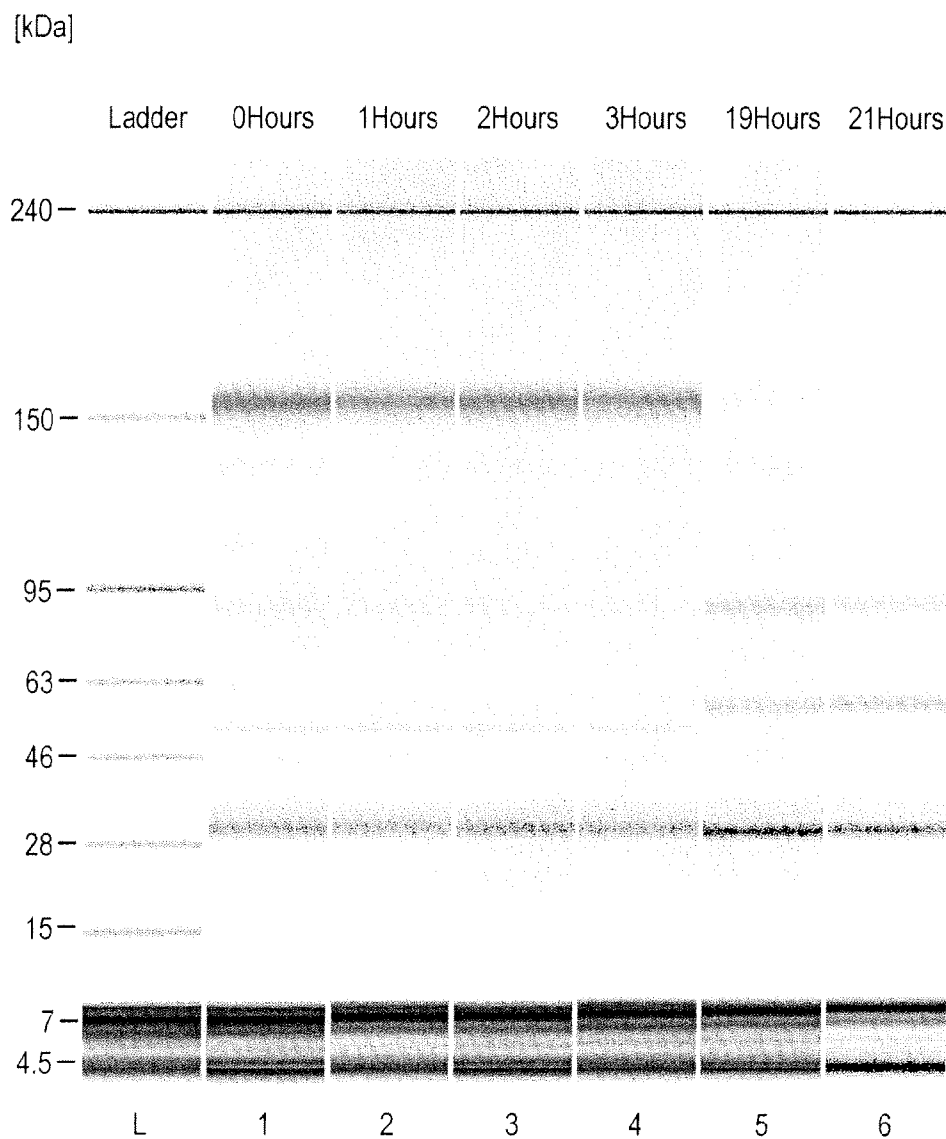
FIG. 15

U.S. Patent

Nov. 5, 2013

Sheet 16 of 40

US 8,574,869 B2



Ocrelizumab Reduction

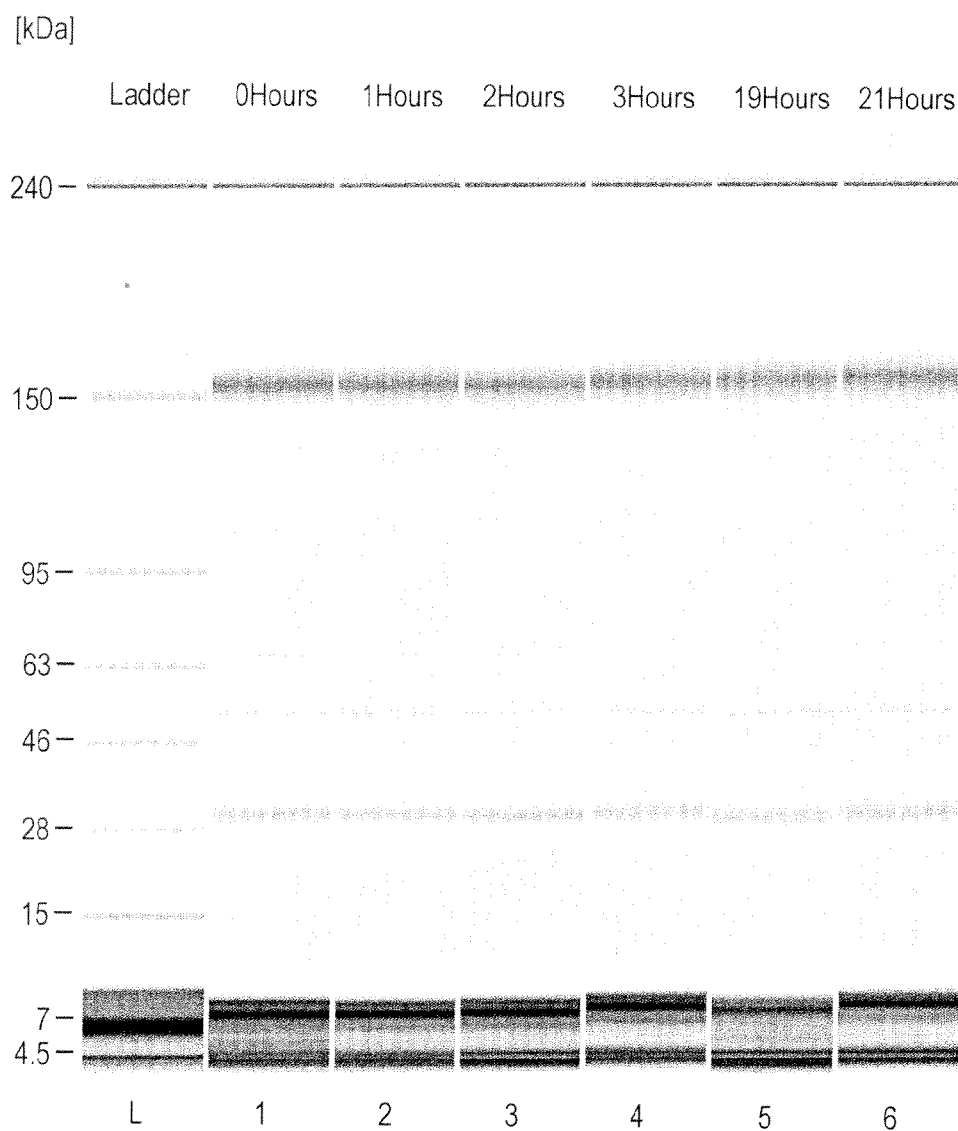
FIG. 16

U.S. Patent

Nov. 5, 2013

Sheet 17 of 40

US 8,574,869 B2



EDTA Inhibits Ocrelizumab Reduction

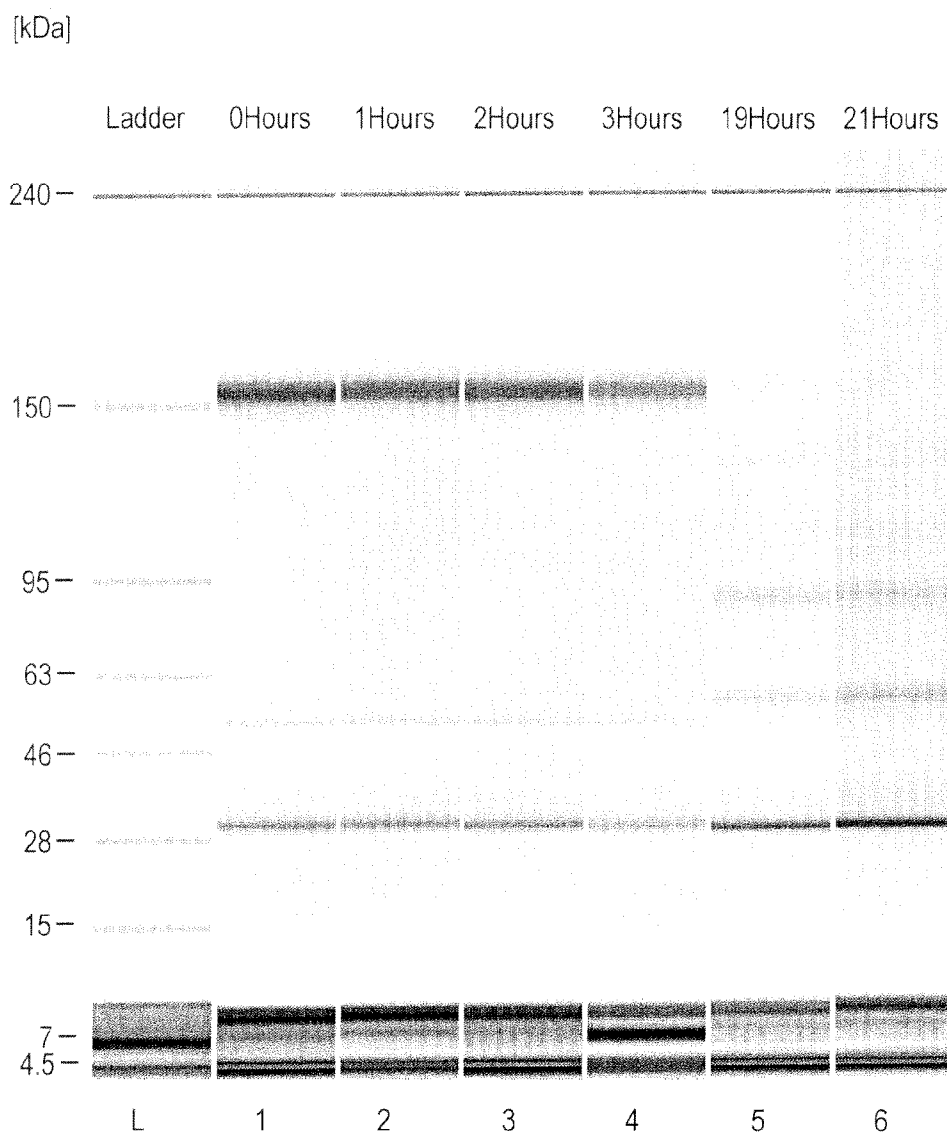
FIG. 17

U.S. Patent

Nov. 5, 2013

Sheet 18 of 40

US 8,574,869 B2



The Lost Reduction Activity in Run 8 HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA

FIG. 18

U.S. Patent

Nov. 5, 2013

Sheet 19 of 40

US 8,574,869 B2

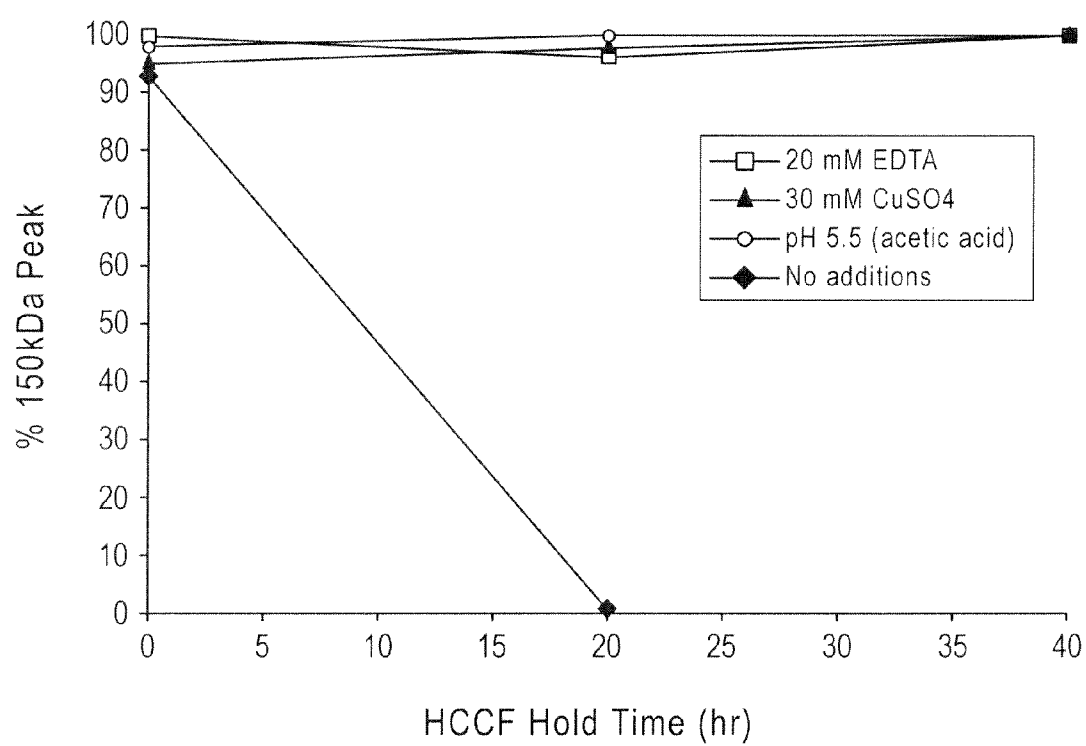


FIG. 19

U.S. Patent

Nov. 5, 2013

Sheet 20 of 40

US 8,574,869 B2

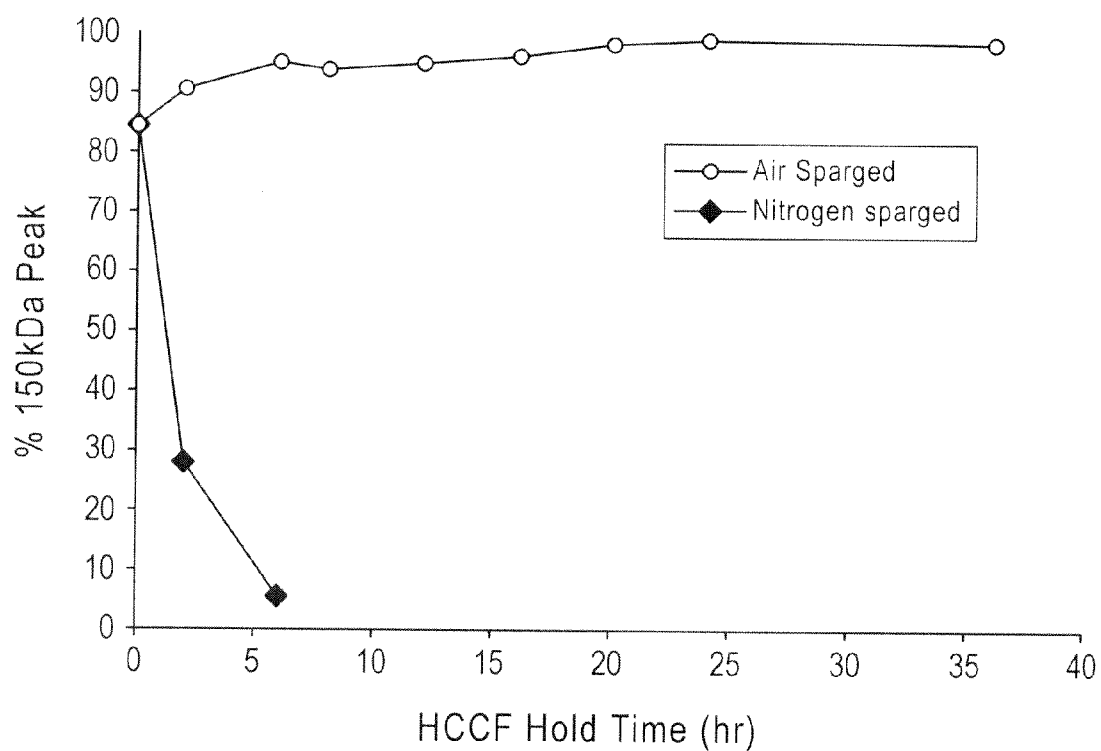


FIG. 20

U.S. Patent

Nov. 5, 2013

Sheet 21 of 40

US 8,574,869 B2

Light Chain

1 15 30 45
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPK
46 60 75 90
LLIYSASFLYSGVPSRFRSGSRSGTDFTLTISLQPEDFATYYCQQ
91 105
HYTTPPTFGQGTKVEIK

FIG. 21

Heavy Chain

1 15 30 45
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGL
46 60 75 90
EWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAED
91 105 120
TAVYYCSRWGGDGFYAMDYWGQGTLLTVSS

FIG. 22

Typical Batch or Fed-Batch Culture Process

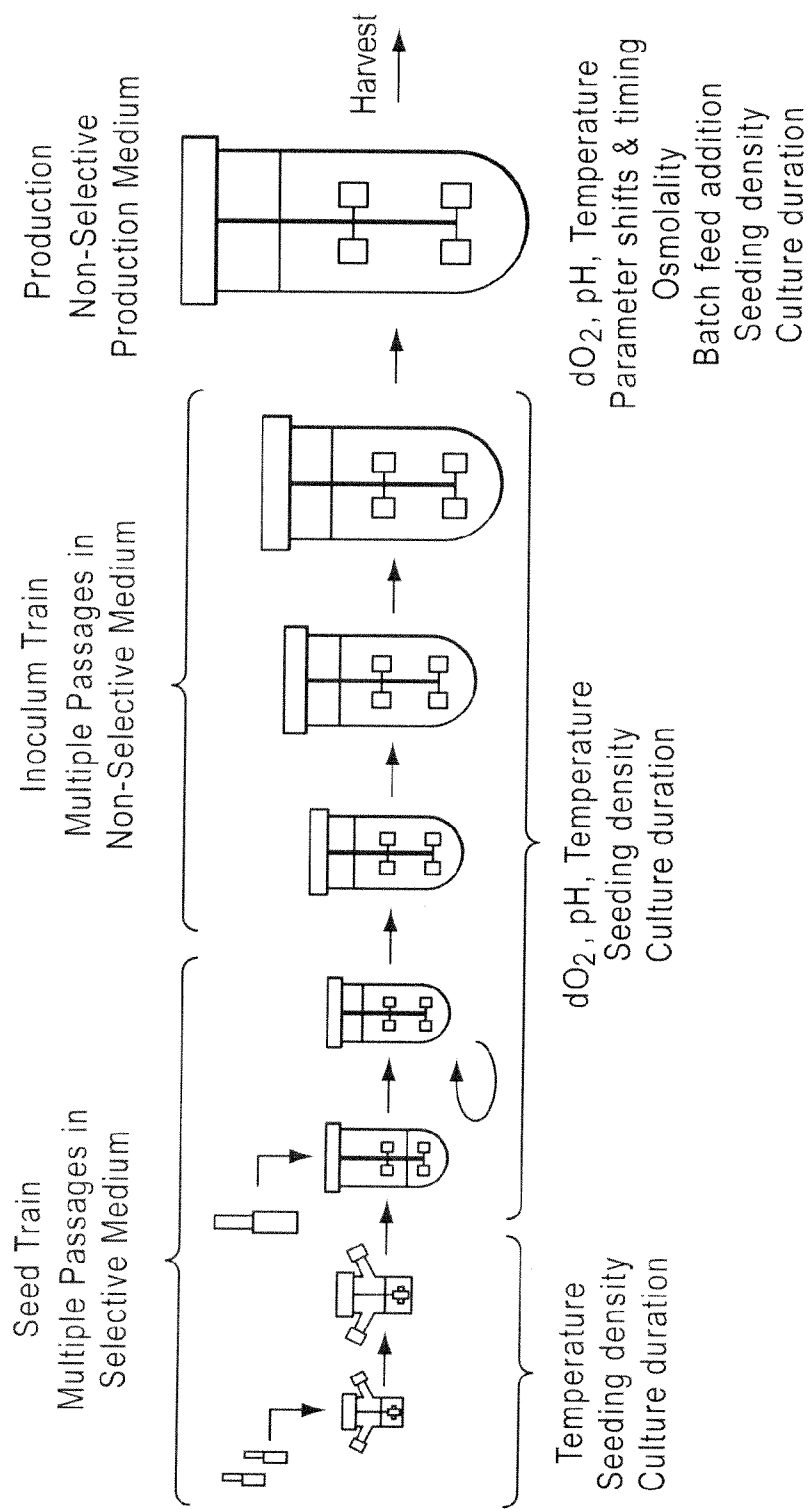


FIG. 23

U.S. Patent

Nov. 5, 2013

Sheet 23 of 40

US 8,574,869 B2

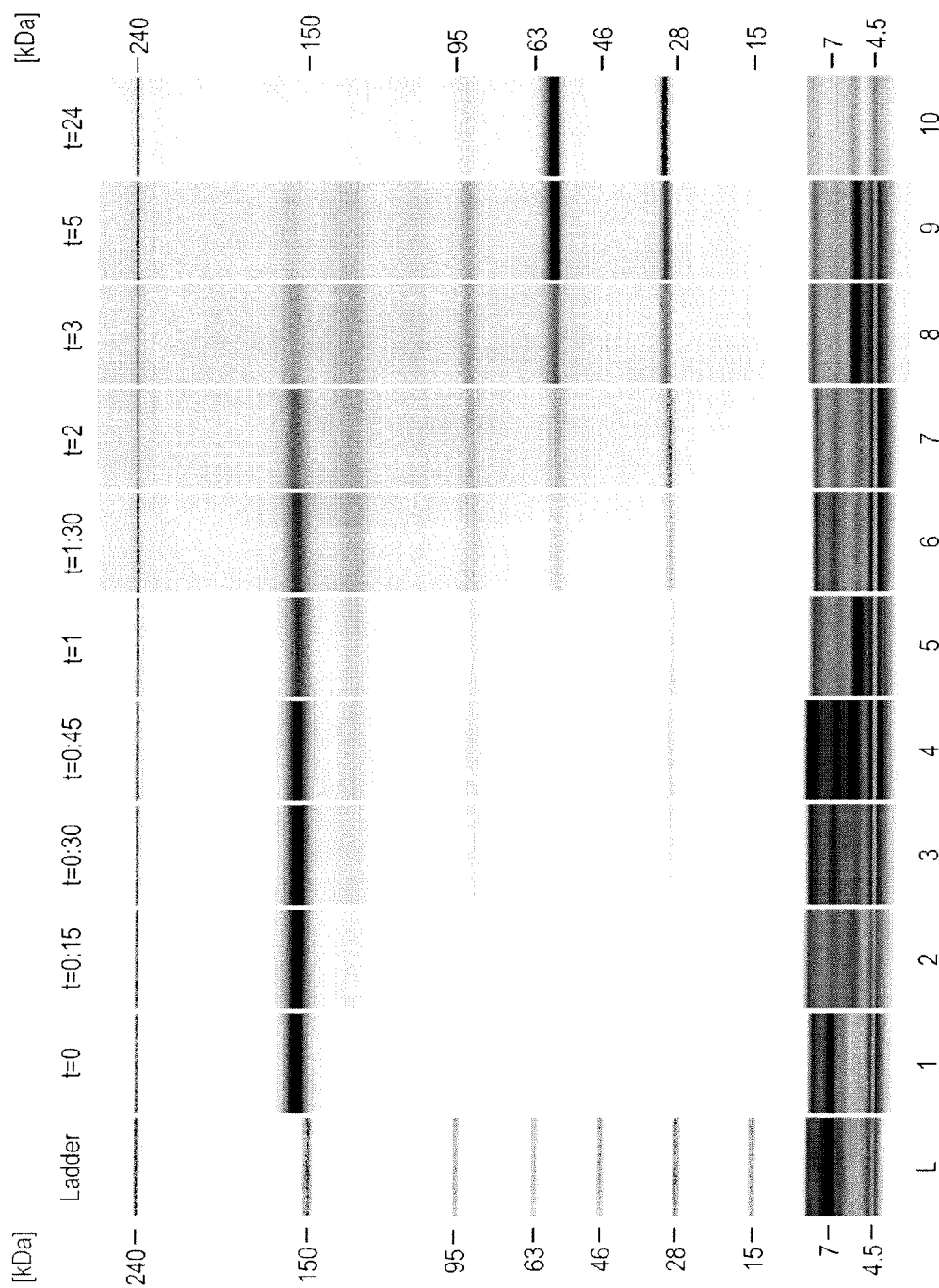


FIG. 24

U.S. Patent

Nov. 5, 2013

Sheet 24 of 40

US 8,574,869 B2

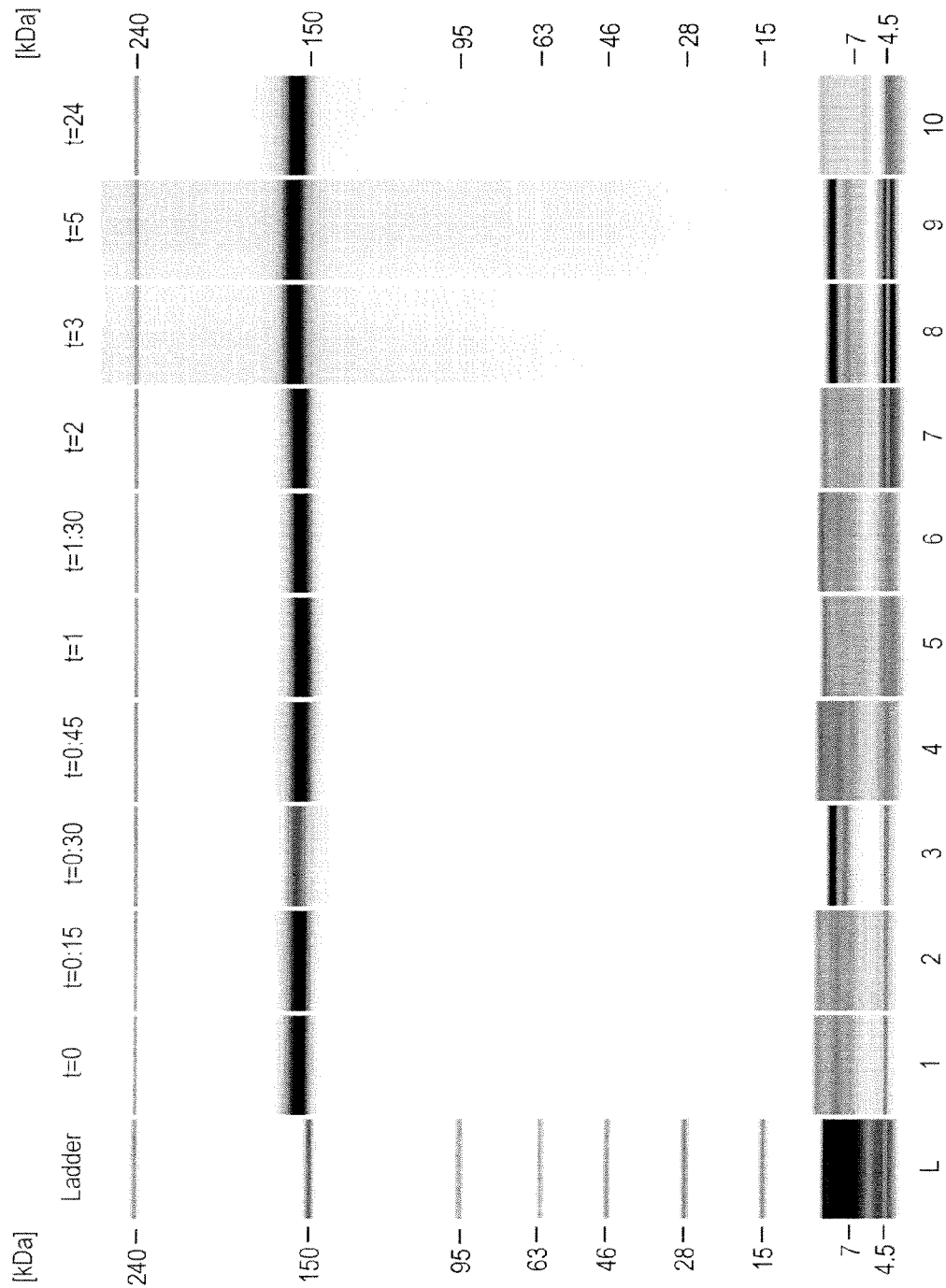


FIG. 25

U.S. Patent

Nov. 5, 2013

Sheet 25 of 40

US 8,574,869 B2

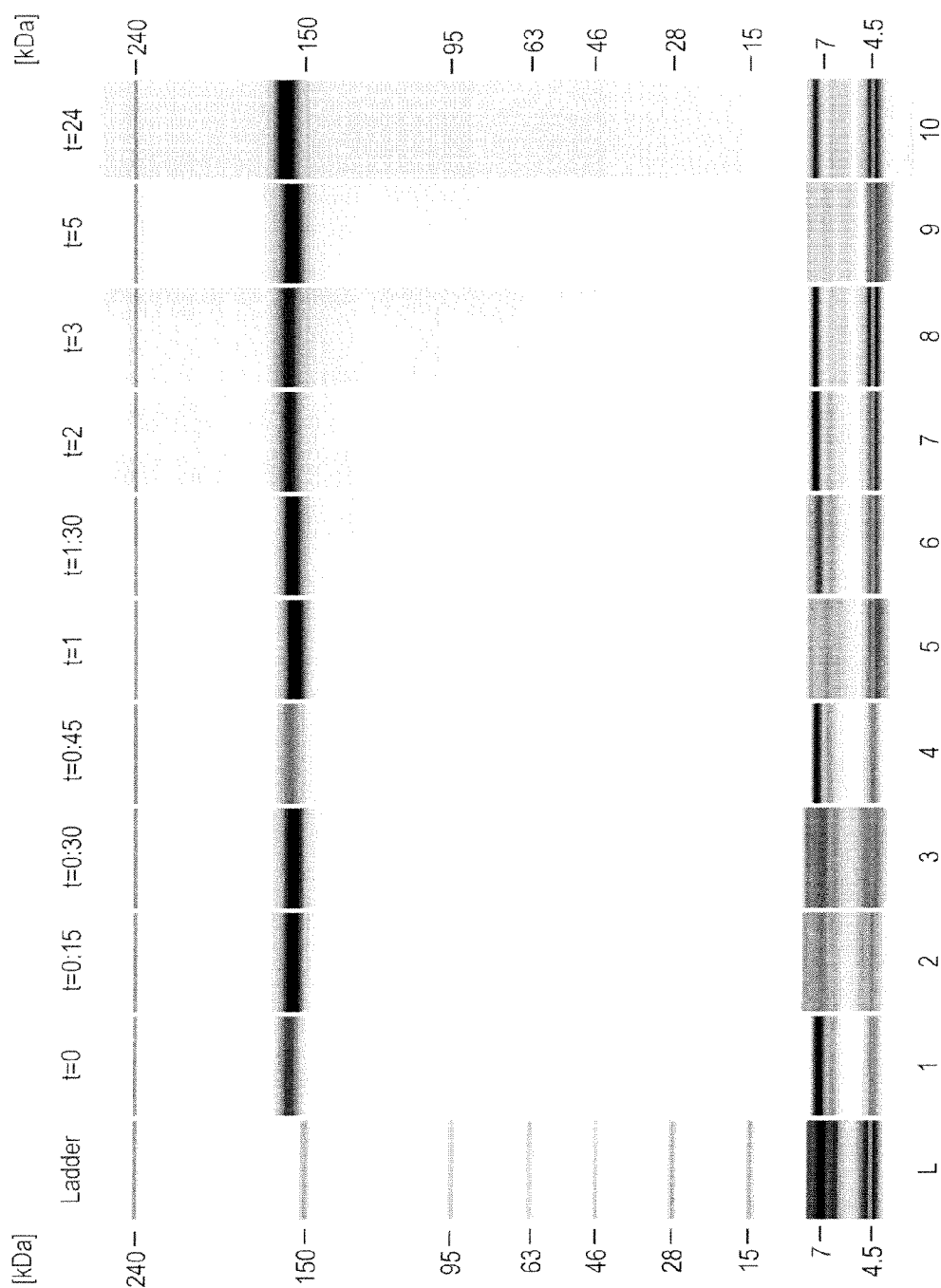


FIG. 26

U.S. Patent

Nov. 5, 2013

Sheet 26 of 40

US 8,574,869 B2

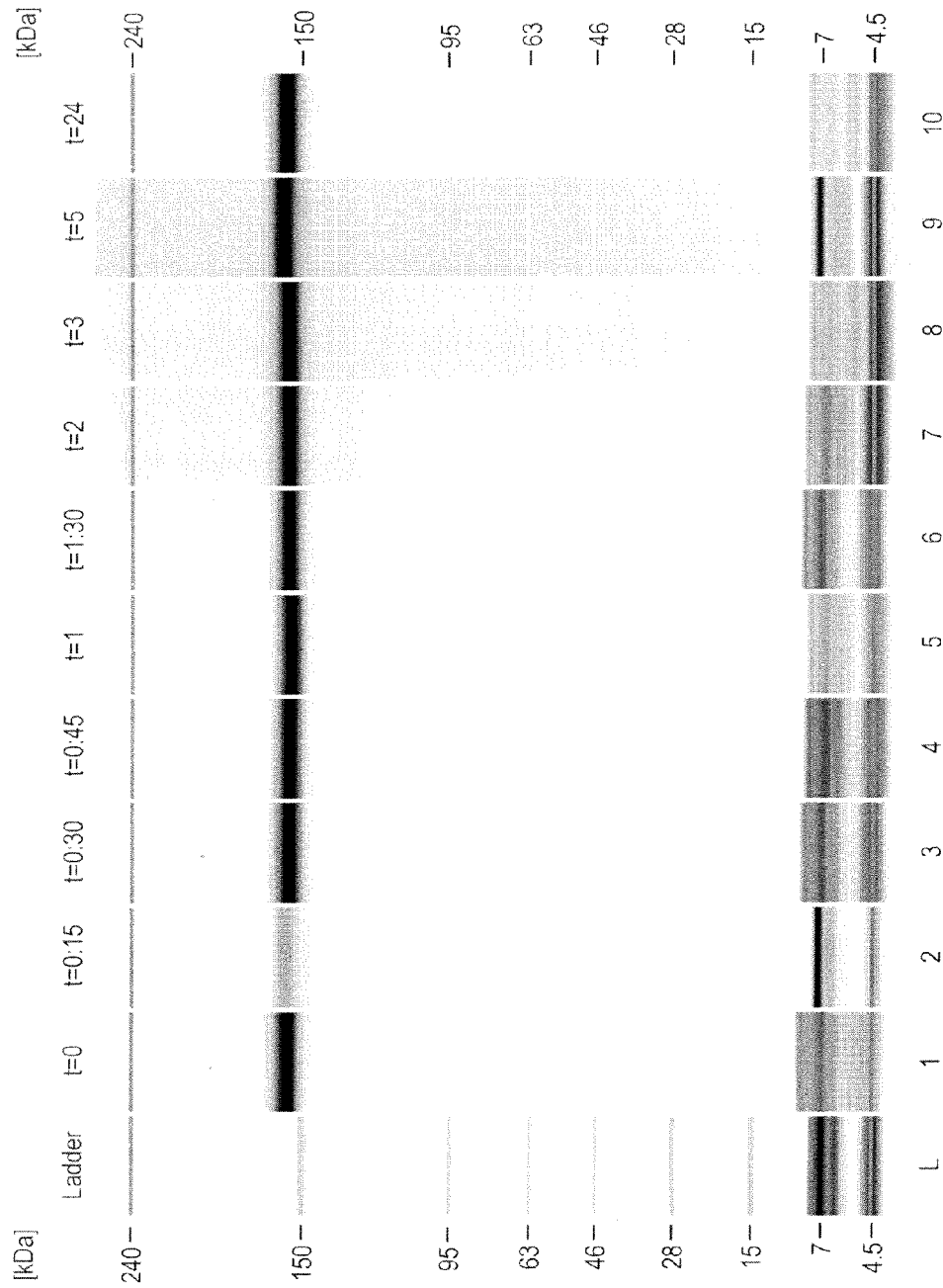


FIG. 27

U.S. Patent

Nov. 5, 2013

Sheet 27 of 40

US 8,574,869 B2

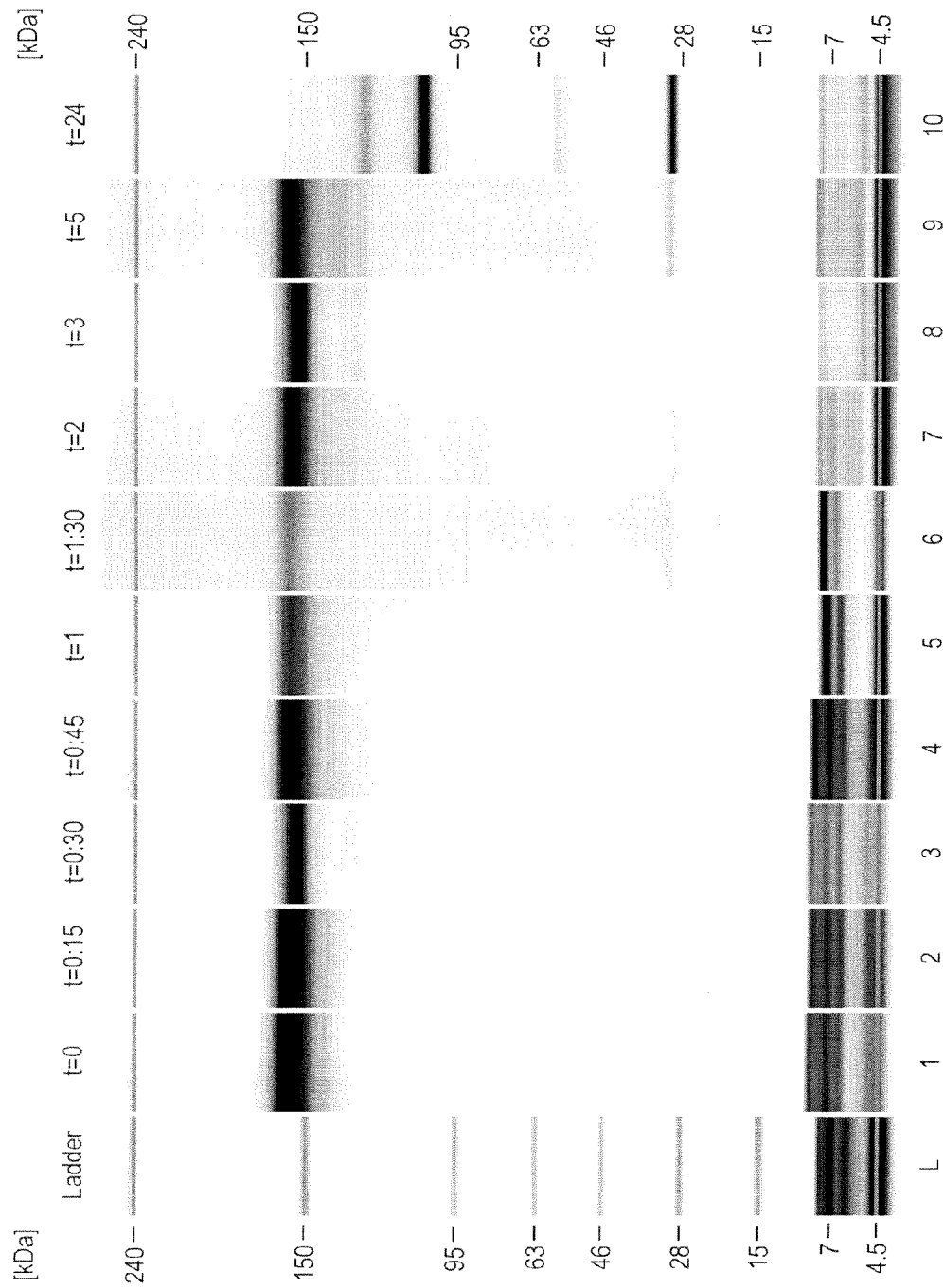


FIG. 28

U.S. Patent

Nov. 5, 2013

Sheet 28 of 40

US 8,574,869 B2

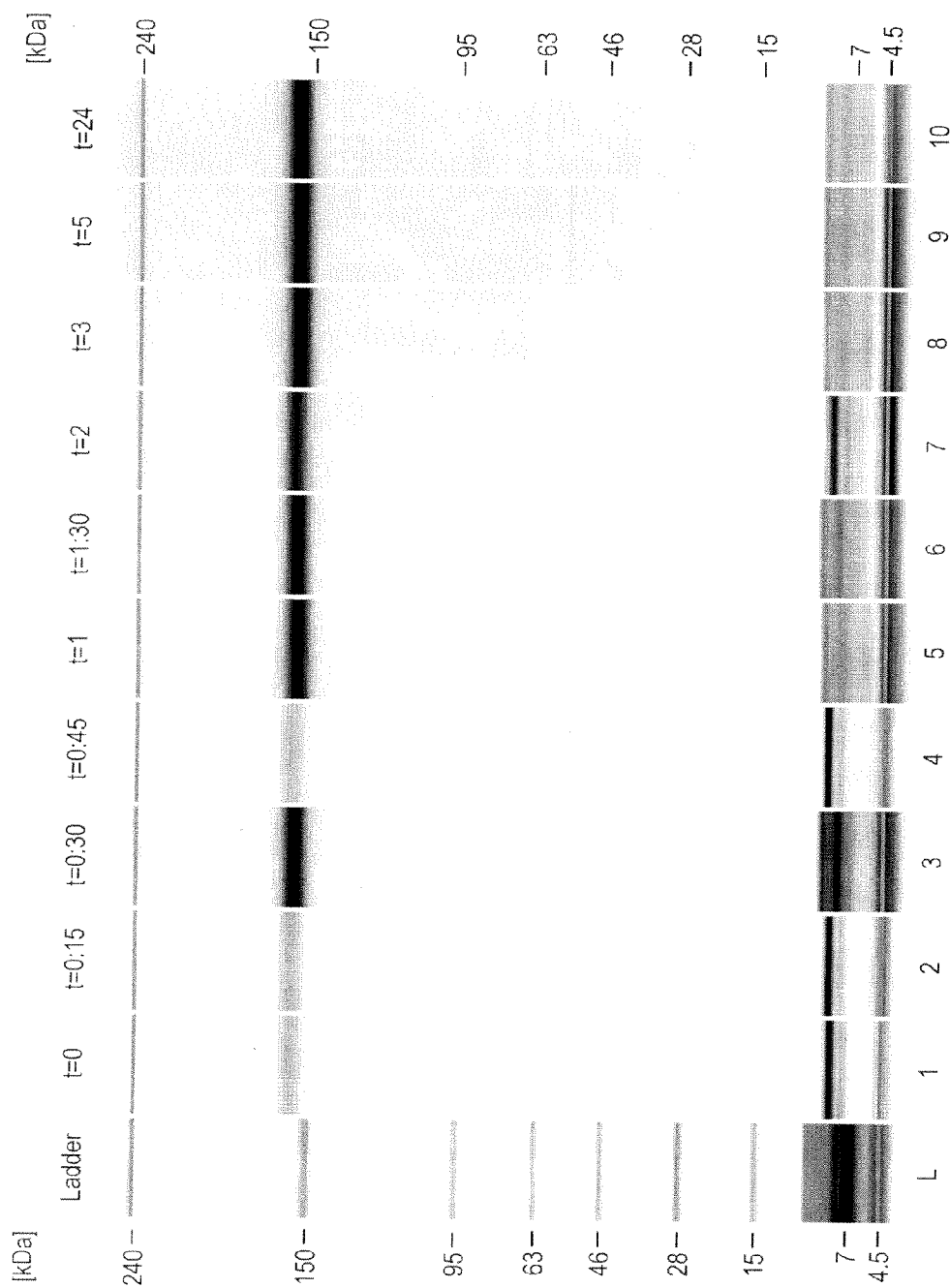


FIG. 29

U.S. Patent

Nov. 5, 2013

Sheet 29 of 40

US 8,574,869 B2

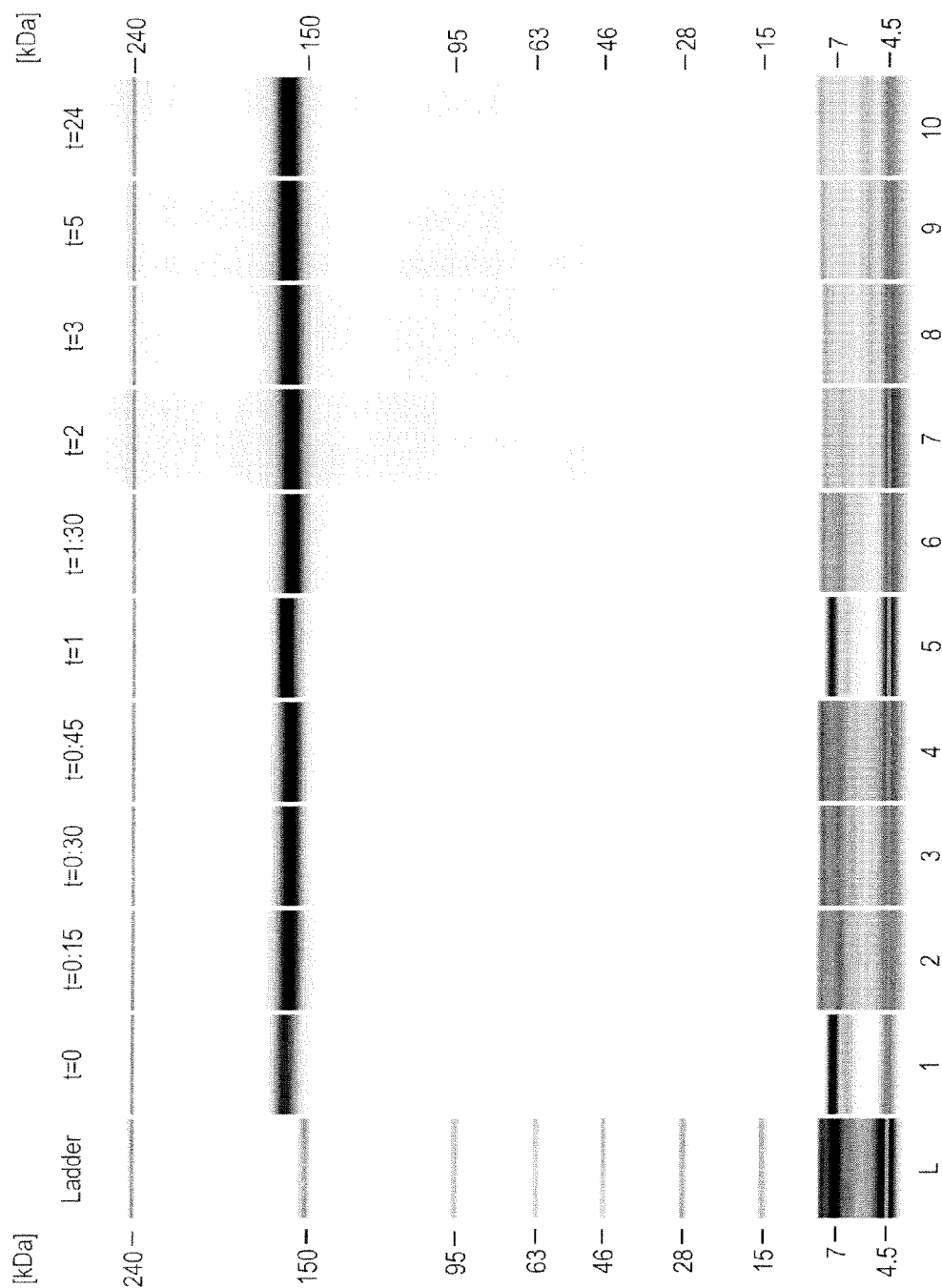


FIG. 30

U.S. Patent

Nov. 5, 2013

Sheet 30 of 40

US 8,574,869 B2

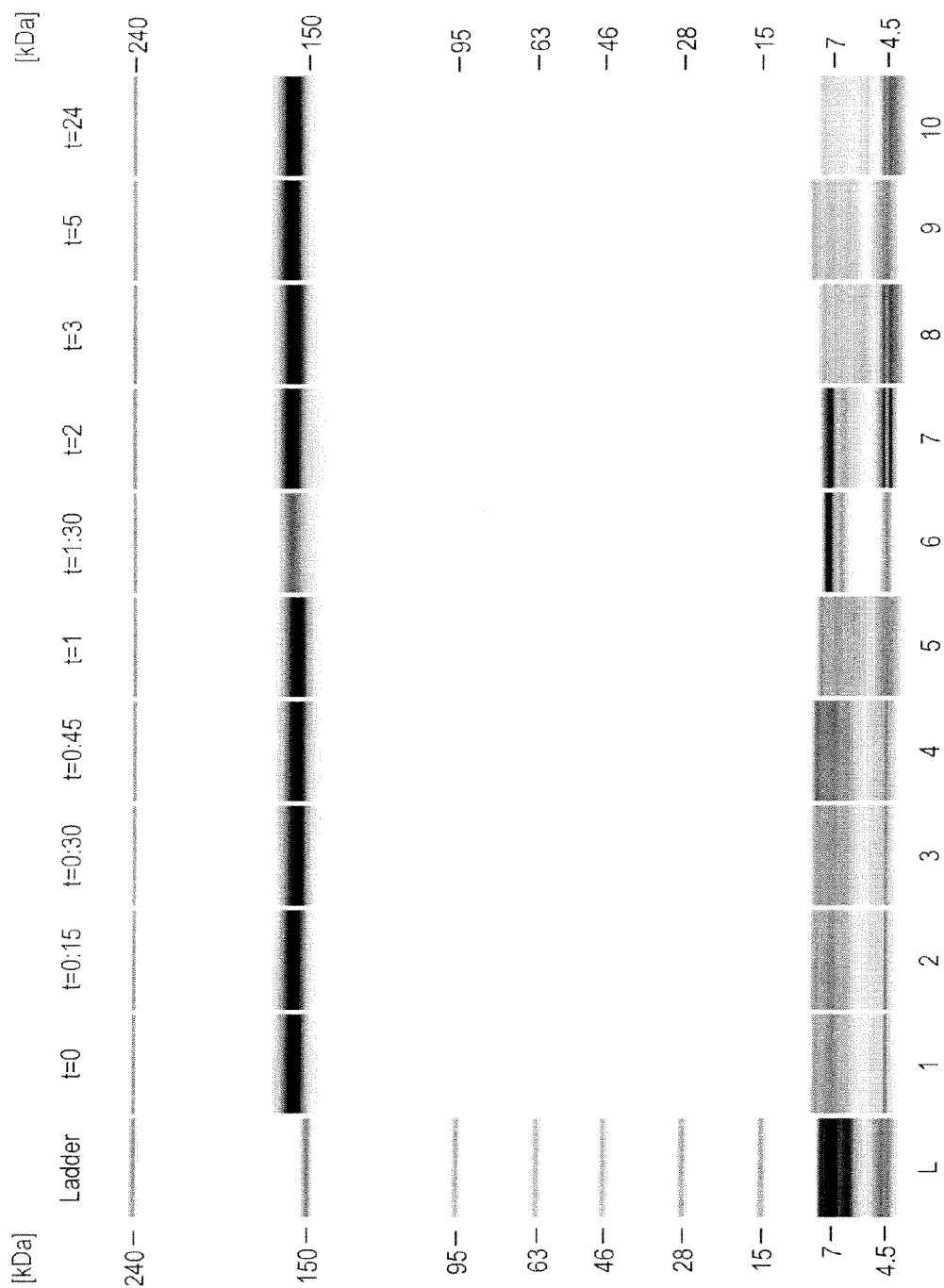


FIG. 31

U.S. Patent

Nov. 5, 2013

Sheet 31 of 40

US 8,574,869 B2

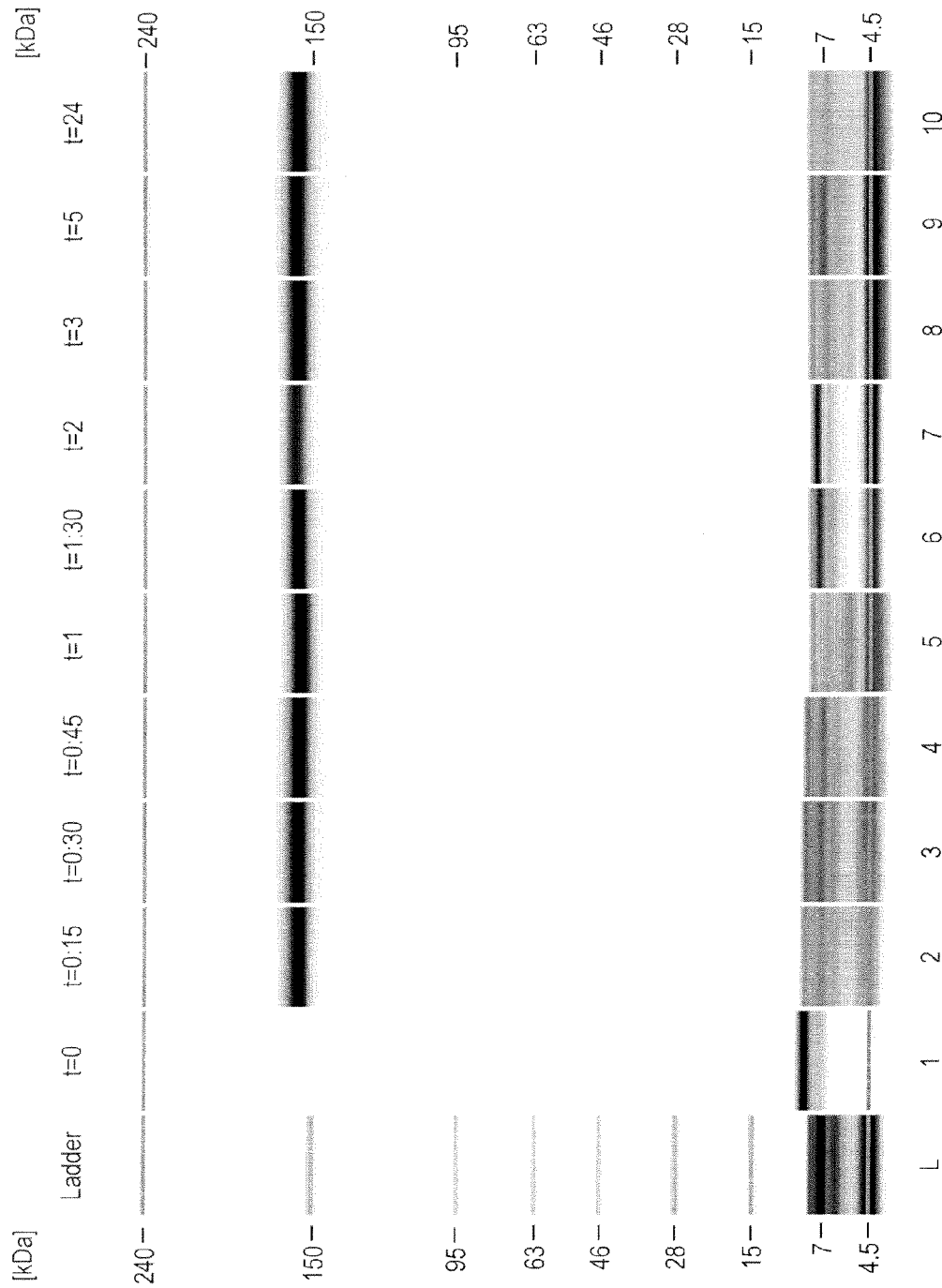


FIG. 32

U.S. Patent

Nov. 5, 2013

Sheet 32 of 40

US 8,574,869 B2

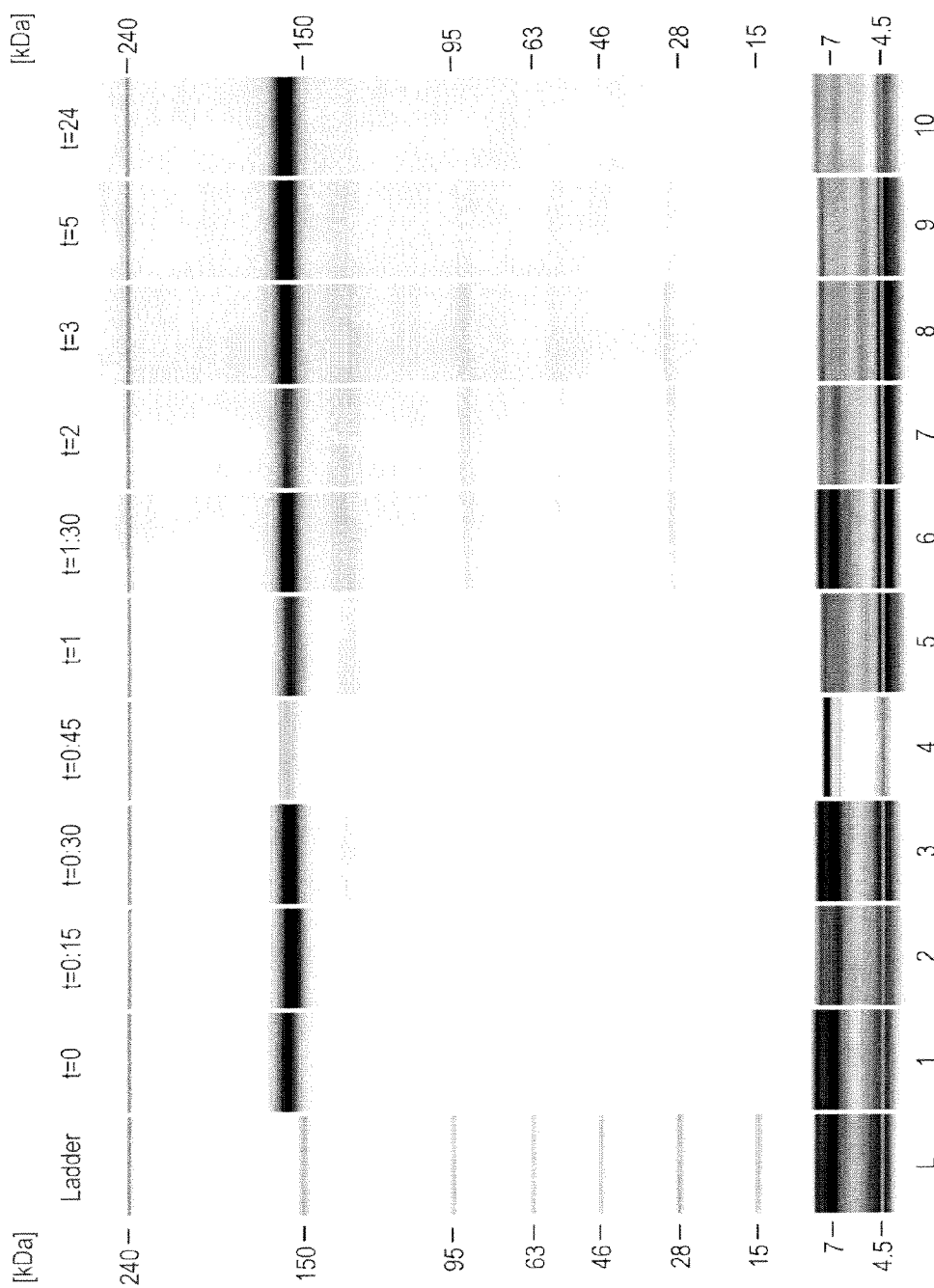


FIG. 33

U.S. Patent

Nov. 5, 2013

Sheet 33 of 40

US 8,574,869 B2

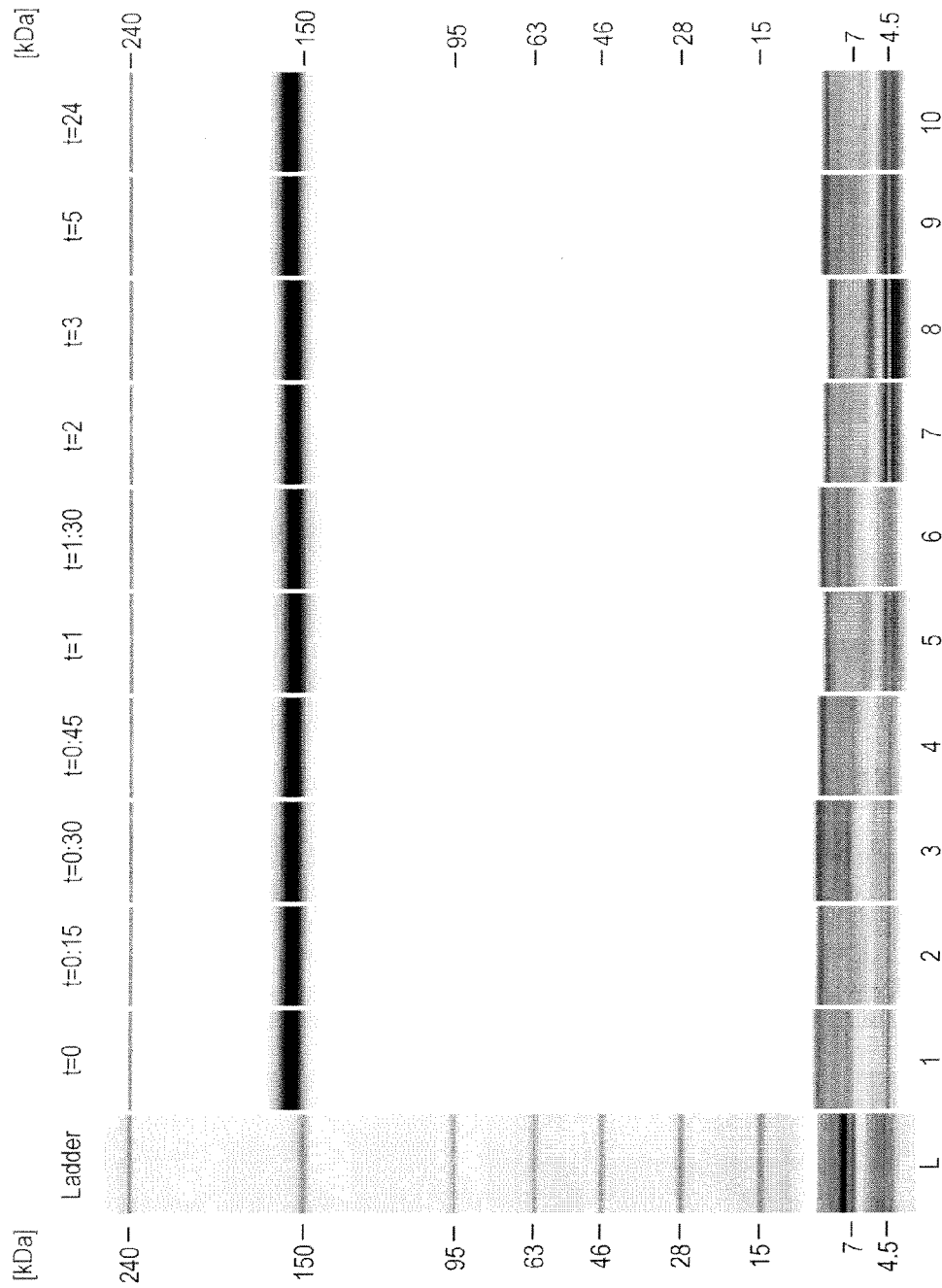


FIG. 34

U.S. Patent

Nov. 5, 2013

Sheet 34 of 40

US 8,574,869 B2

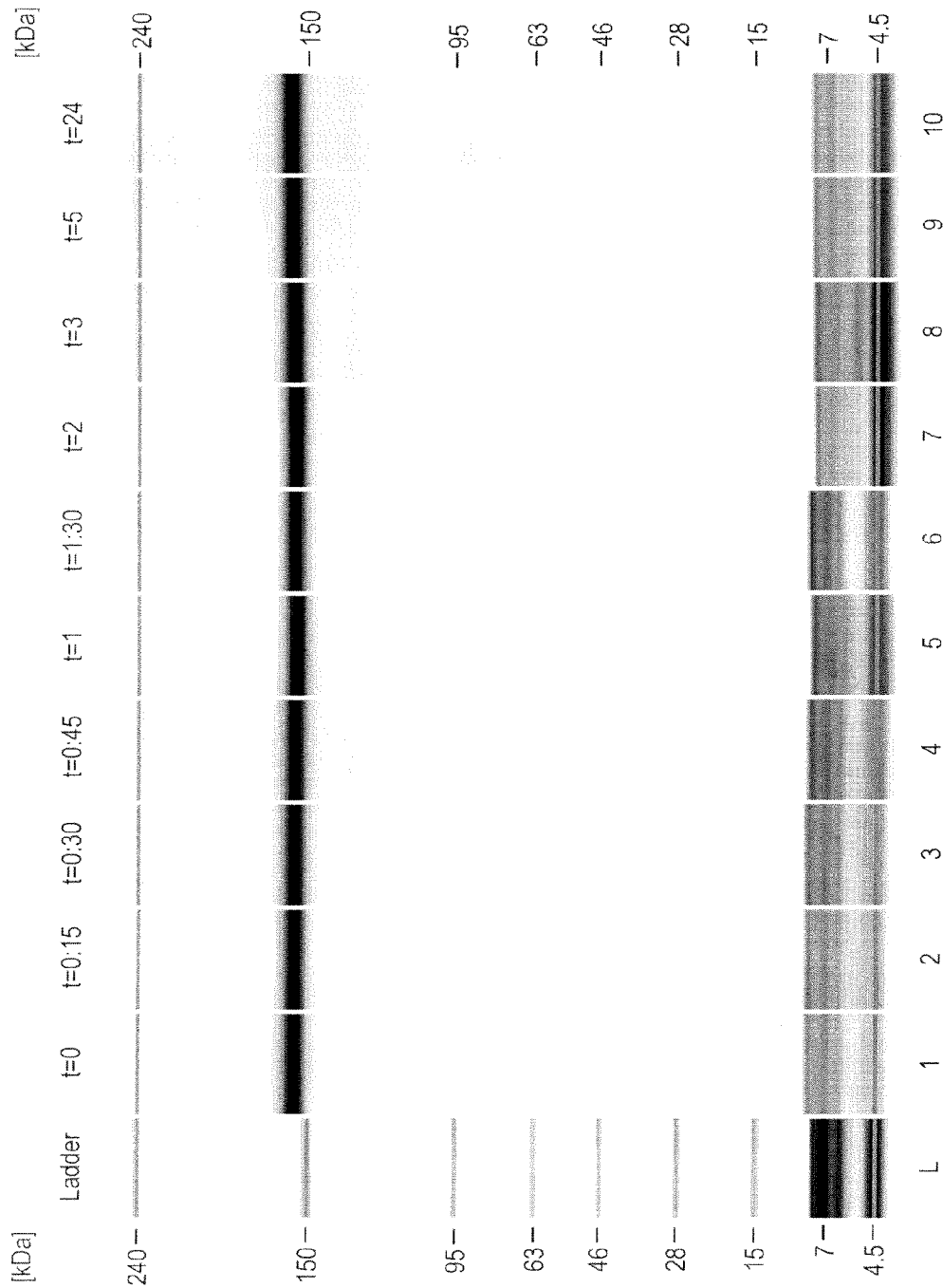


FIG. 35

U.S. Patent

Nov. 5, 2013

Sheet 35 of 40

US 8,574,869 B2

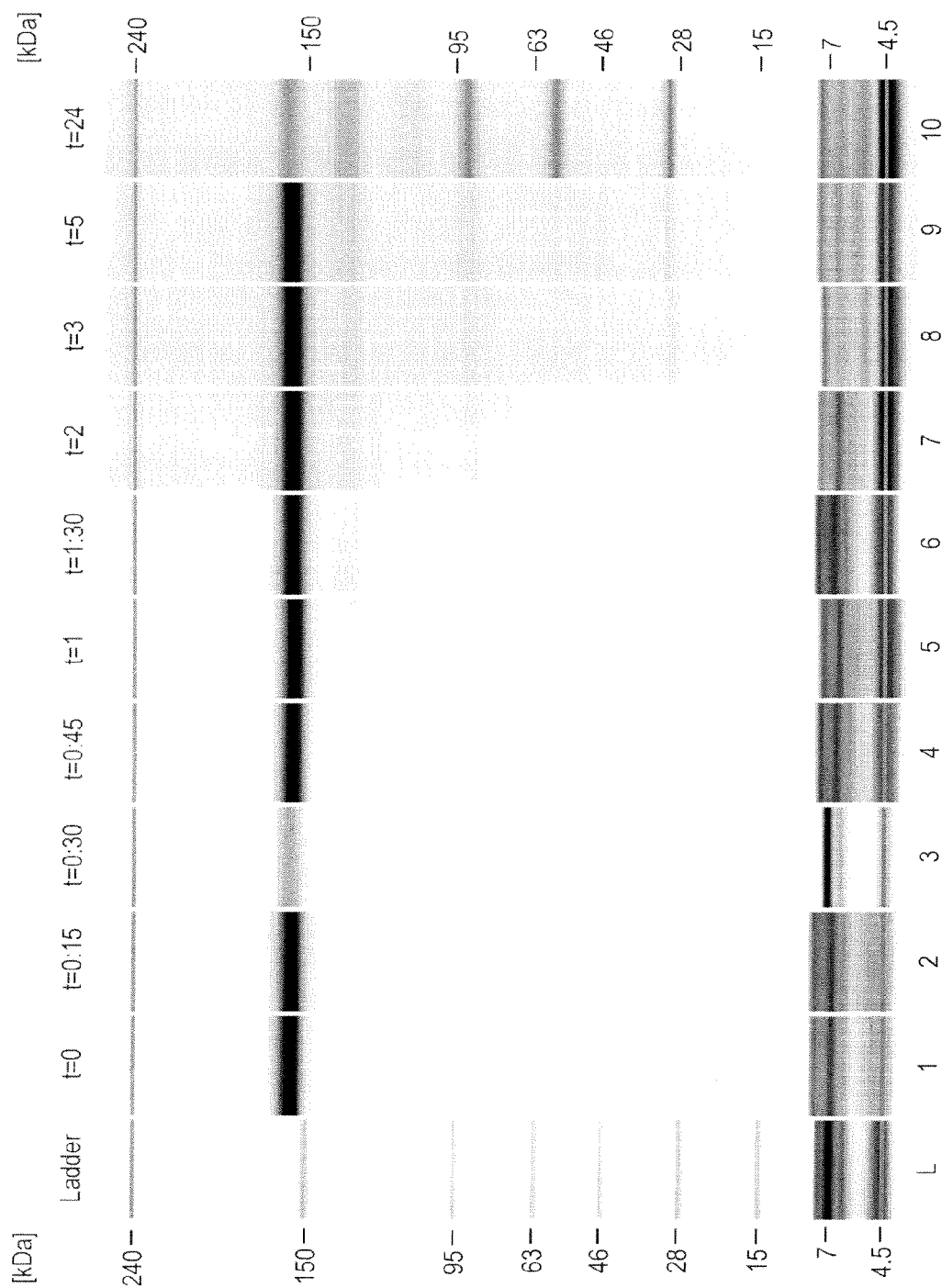


FIG. 36

U.S. Patent

Nov. 5, 2013

Sheet 36 of 40

US 8,574,869 B2

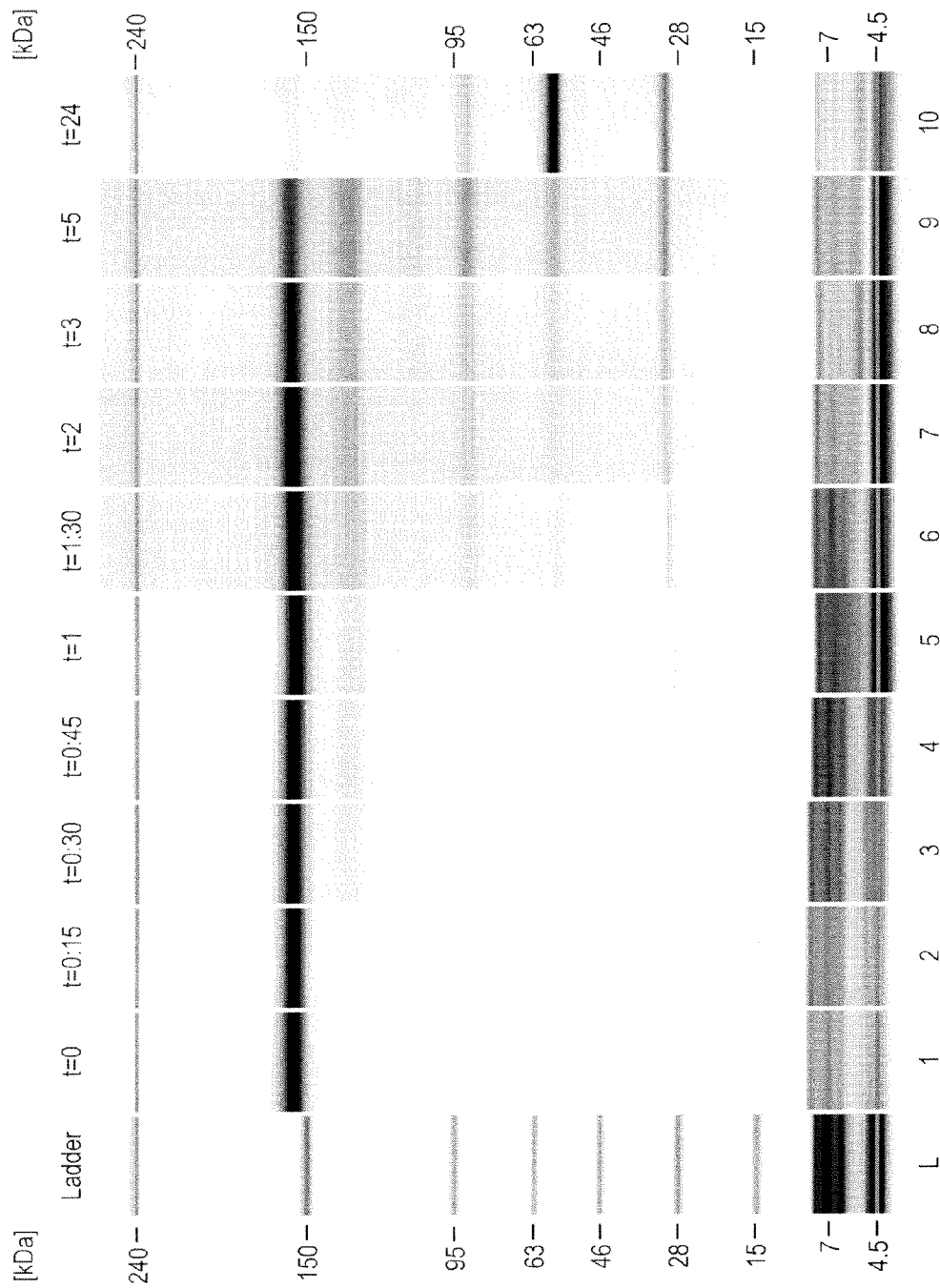


FIG. 37

U.S. Patent

Nov. 5, 2013

Sheet 37 of 40

US 8,574,869 B2

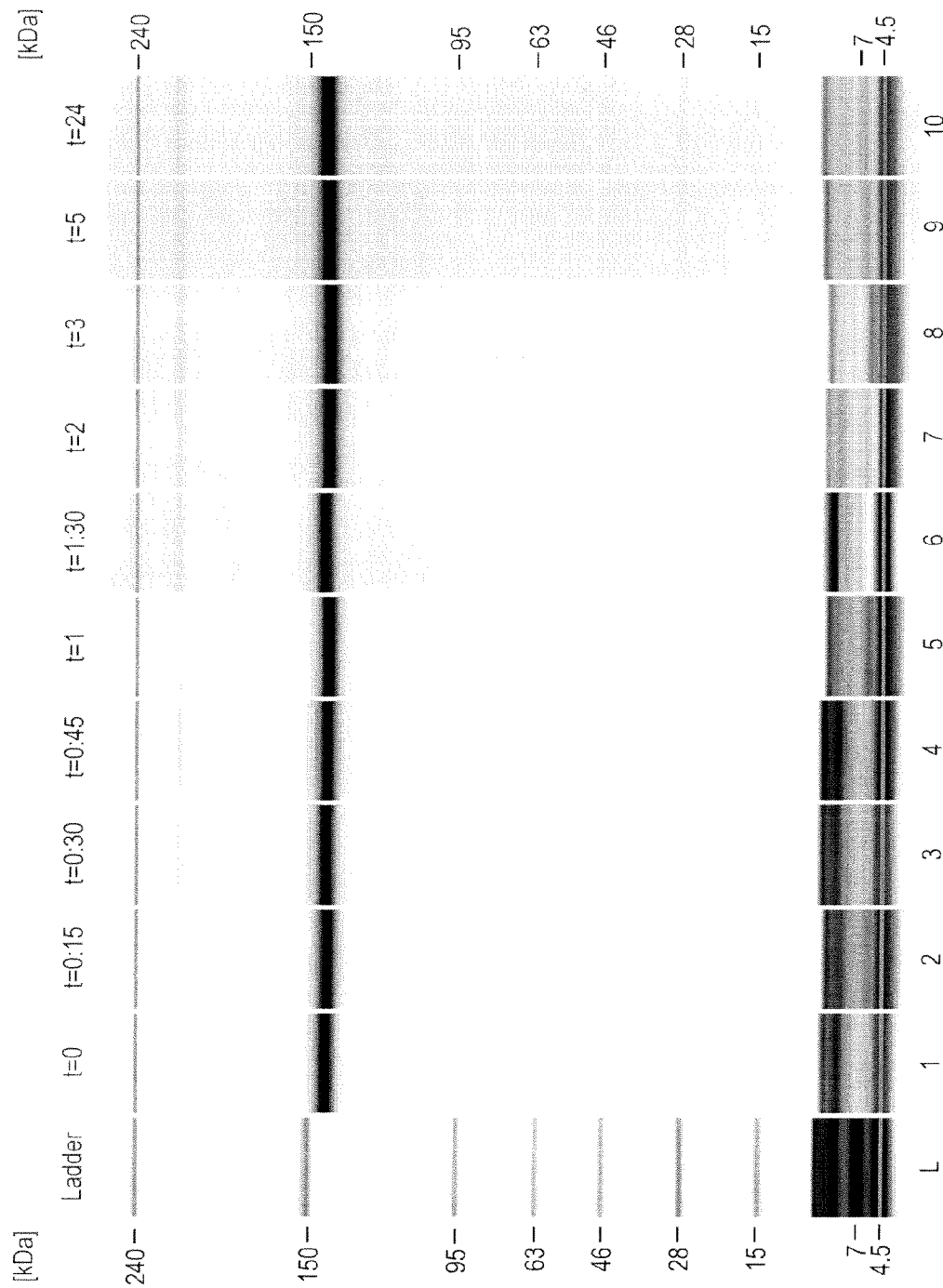


FIG. 38

U.S. Patent

Nov. 5, 2013

Sheet 38 of 40

US 8,574,869 B2

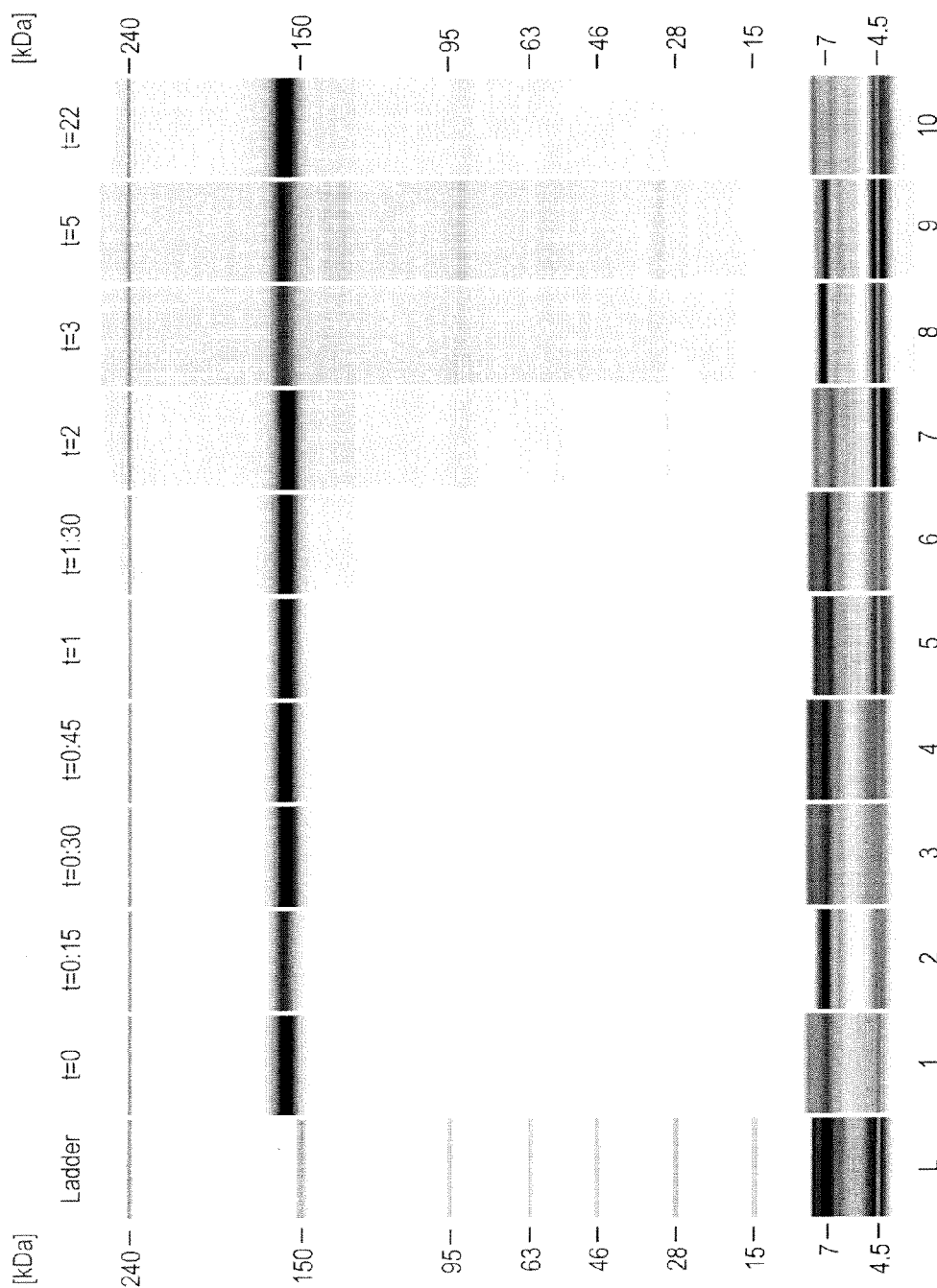


FIG. 39

U.S. Patent

Nov. 5, 2013

Sheet 39 of 40

US 8,574,869 B2

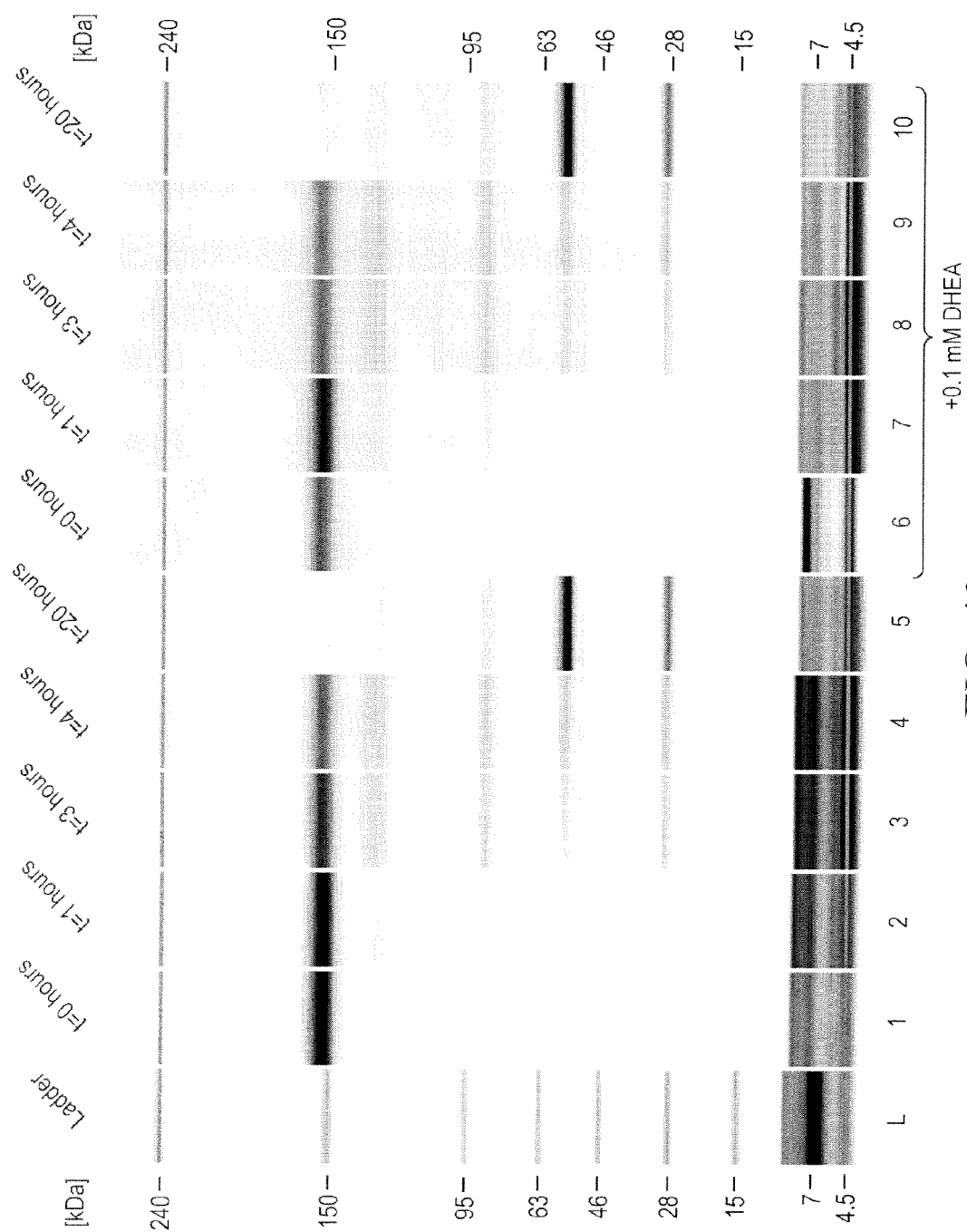


FIG. 40

U.S. Patent

Nov. 5, 2013

Sheet 40 of 40

US 8,574,869 B2

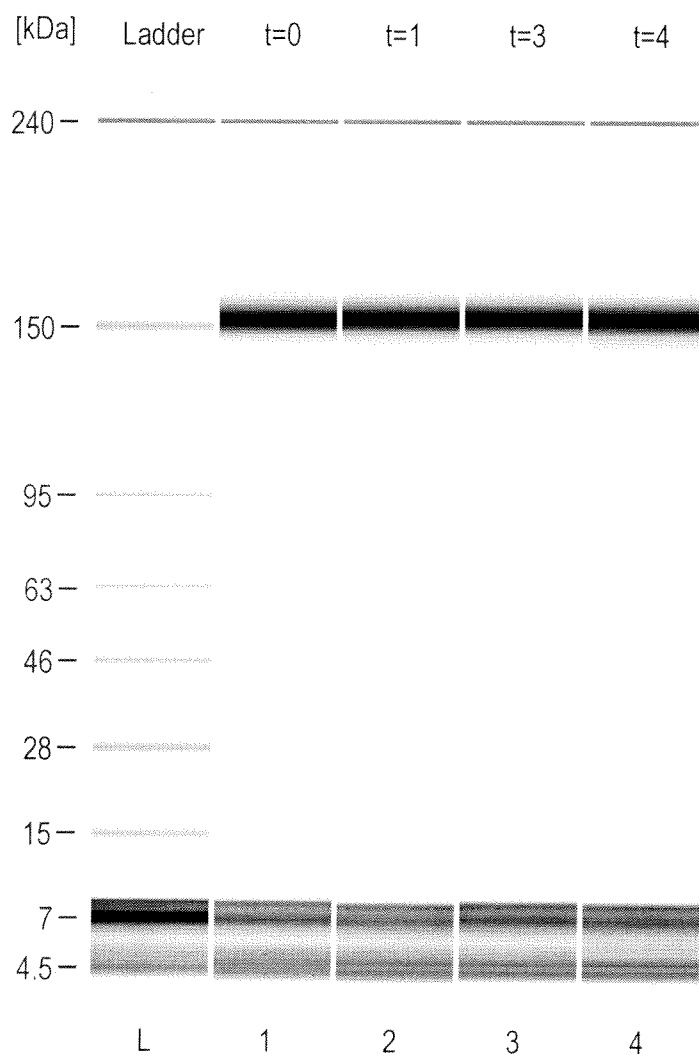


FIG. 41

US 8,574,869 B2

1

PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/217,745, filed Jul. 8, 2008, which is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional Application No. 60/948,677 filed Jul. 9, 2007, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention concerns methods and means for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

BACKGROUND OF THE INVENTION

In the biotechnology industry, pharmaceutical applications require a variety of proteins produced using recombinant DNA techniques. Generally, recombinant proteins are produced by cell culture, using either eukaryotic cells, such as mammalian cells, or prokaryotic cells, such as bacterial cells, engineered to produce the protein of interest by insertion of a recombinant plasmid containing the nucleic acid encoding the desired protein. For a protein to remain biologically active, the conformation of the protein, including its tertiary structure, must be maintained during its purification and isolation, and the protein's multiple functional groups must be protected from degradation.

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells, such as the PER.C6® cell line isolated from a human retinal cell, which provides human glycosylation characteristics, and is able to naturally produce antibodies that match human physiology, have been approved by regulatory agencies for the production of biopharmaceutical products.

Usually, to begin the production cycle, a small number of transformed recombinant host cells are allowed to grow in culture for several days (see, e.g., FIG. 23). Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to

2

secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Typically, harvesting includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, the production of recombinant proteins is still not without difficulties. Thus, for example, during the recombinant production of polypeptides comprising disulfide bonds, especially multi-chain polypeptides comprising inter-chain disulfide bonds such as antibodies, it is essential to protect and retain the disulfide bonds throughout the manufacturing, recovery and purification process, in order to produce properly folded polypeptides with the requisite biological activity.

SUMMARY OF THE INVENTION

The instant invention generally relates to a method for preventing reduction of a disulfide bond in a polypeptide expressed in a recombinant host cell, comprising supplementing the pre-harvest or harvested culture fluid of the recombinant host cell with an inhibitor of thioredoxin or a thioredoxin-like protein.

In one embodiment, the thioredoxin inhibitor is added to the pre-harvest culture fluid.

In another embodiment, the thioredoxin inhibitor is added to the harvested culture fluid.

In a further embodiment, the thioredoxin inhibitor is a direct inhibitor of thioredoxin.

In all embodiments, the thioredoxin inhibitor may, for example, be an alkyl-2-imidazolyl disulfide or a naphthoquinone spiroketal derivative.

In a further embodiment, the thioredoxin inhibitor is a specific inhibitor of thioredoxin reductase.

In a still further embodiment, the thioredoxin inhibitor is a gold complex, where the gold complex may, for example, be aurothioglucose (ATG) or aurothiomalate (ATM). While the effective inhibitory concentration may vary, it typically is between about 0.1 mM and 1 mM. Similarly, the minimum effective inhibitory concentration varies depending on the nature of the polypeptide and overall circumstances, and is typically reached when the ATG or ATM concentration is at least about four-times of thioredoxin concentration in the pre-harvest or harvested culture fluid.

In another embodiment of this aspect of the invention, the thioredoxin inhibitor is a metal ion, where the metal ion, without limitation, may be selected from the group consisting of Hg^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+} , and Mn^{2+} . When the metal ion is added in the form of cupric sulfate, the effective inhibitory concentration generally is between about 5 μM and about 100 μM , or between about 10 μM and about 80 μM , or between about 15 μM and about 50 μM . The minimum inhibitory concentration of cupric sulfate also varies, but typically is reached when cupric sulfate is added at a concentration at least about two-times of thioredoxin concentration in the pre-harvest or harvested culture fluid.

In different embodiment, the thioredoxin inhibitor is an oxidizing agent, e.g., an inhibitor of G6PD, such as, for example, pyridoxal 5'-phosphate, 1 fluoro-2,4 dinitrobenzene, dehydroepiandrosterone (DHEA) or epiandrosterone (EA); cystine or cysteine. Typical effective inhibitor concen-

US 8,574,869 B2

3

trations of DHEA are between about 0.05 mM and about 5 mM, or between about 0.1 mM and about 2.5 mM.

In a further embodiment, the thioredoxin inhibitor is an inhibitor of hexokinase activity, including, without limitation, chelators of metal ions, such as, for example, ethylenediamine tetraacetic acid (EDTA). EDTA is typically added and effective at a concentration between about 5 mM and about 60 mM, or about 10 mM and about 50 mM, or about 20 mM and about 40 mM.

In other preferred embodiments, the inhibitor of hexokinase activity is selected from the group consisting of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, and lyxose.

Other inhibitors include cystine, cysteine, and oxidized glutathione which are typically added at a concentration at least about 40-times of the concentration of the polypeptide in question in the pre-harvest or harvested culture fluid.

In a still further embodiment, the thioredoxin inhibitor is an siRNA, an antisense nucleotide, or an antibody specifically binding to a thioredoxin reductase.

In another embodiment, the thioredoxin inhibitor is a measure indirectly resulting in the inhibition of thioredoxin activity. This embodiment includes, for example, air sparging the harvested culture fluid of the recombinant host cell, and/or lowering the pH of the harvested culture fluid of the recombinant host cell.

In various embodiments, indirect means for inhibiting thioredoxin activity, such as air sparging and/or lowering of the pH, can be combined with the use of direct thioredoxin inhibitors, such as those listed above.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', F(ab')₂, scFv, (scFv)₂, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha_4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti- $\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In a further embodiment, the HER receptor is HER1 and/or HER2, preferably HER2. The HER2 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NO: 16, 17, 18, and 19.

4

In another embodiment, the therapeutic antibody is an antibody that binds to CD20. The anti-CD20 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 1 through 15.

In yet another embodiment, the therapeutic antibody is an antibody that binds to VEGF. The anti-VEGF antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 20 through 25.

In an additional embodiment, the therapeutic antibody is an antibody that binds CD11a. The anti-CD11a antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 26 through 29.

In a further embodiment, the therapeutic antibody binds to a DR5 receptor. The anti-DR5 antibody may, for example, be selected from the group consisting of Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, and preferably is Apomab 8.3 or Apomab 7.3, and most preferably Apomab 7.3.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellierian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an

US 8,574,869 B2

5

ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell, such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

In all embodiments, the recombinant host cell can also be a prokaryotic host cell, such as a bacterial cell, including, without limitation, *E. coli* cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) demonstrating that ocrelizumab (rhuMab 2H7—Variant A) inside the dialysis bag remained intact during the incubation period.

FIG. 2. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab outside the dialysis bag was reduced during the incubation period. This is evidenced by the loss of intact antibody (~150 kDa) and the formation of antibody fragments depicted in the Figure. At the 48-hour time point (Lane 7), the reduced antibody appeared to be reoxidized, presumably as a result of losing reduction activity in the Harvested Cell Culture Fluid (HCCF). The band appearing just above the 28 kDa marker arose from the light chain of antibody. There was a significant amount of free light already present in the HCCF before the incubation began. The presence of excess free light chain and dimers of light chain in the HCCF is typical for the cell line producing ocrelizumab.

FIG. 3. Free Thiol Levels from Dialysis Experiment: Purified ocrelizumab in phosphate buffered saline (PBS) was inside the dialysis bag and HCCF containing ocrelizumab was outside the bag. Free thiols inside (boxes) and outside (diamonds) the dialysis bag reached comparable levels within a few hours, indicating a good exchange of small molecule components in the HCCF between inside and outside the dialysis bag.

FIG. 4. Thioredoxin System and Other Reactions Involved in Antibody Reduction: The thioredoxin system, comprising thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, functions as a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis.

FIG. 5. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) demonstrating that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in PBS resulted in the complete reduction of ocrelizumab; the ocrelizumab was completely reduced in less than 21 hours.

FIG. 6. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose: The addition of aurothioglucose (ATG) to the same reaction mixture as described in the caption for FIG. 5, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. 7. In vitro Activity of Thioredoxin System Inhibited by Aurothiomalate: The addition of aurothiomalate (ATM) at a concentration of 1 mM to the same reaction mixture as

6

described in the caption for FIG. 5, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. 8. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in 10 mM histidine sulfate buffer resulted in the reduction of ocrelizumab in less than 1 hour.

FIG. 9. In vitro Activity of Thioredoxin System Inhibited by CuSO₄: The addition of CuSO₄ at a concentration of 50 μ M to the same reaction mixture as described in the caption for FIG. 8 effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. 10. Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab was reduced in an incubation experiment using HCCF from a homogenized CCF generated from a 3-L fermentor.

FIG. 11. Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the addition of 1 mM aurothioglucose to the same HCCF as used for the incubation experiment as shown in FIG. 10 inhibited the reduction of ocrelizumab.

FIG. 12. Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate: Digital gel-like image from Bioanalyzer (each lane representing a time point) analysis indicating that the addition of 1 mM aurothiomalate to the same HCCF as used for the incubation experiment shown in FIG. 10 inhibited the reduction of ocrelizumab.

FIG. 13. Losing Reduction Activity in HCCF: The HCCF from one of the large scale manufacturing runs for ocrelizumab (the "beta" run) that was subject to several freeze/thaw cycles demonstrated no ocrelizumab reduction when used in an incubation experiment. This was shown by Bioanalyzer analysis (each lane representing a time point), and can be contrasted to the antibody reduction seen previously in the freshly thawed HCCF from the same fermentation batch.

FIG. 14. The Lost Reduction Activity in HCCF Restored by Addition of NADPH: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of NADPH at a concentration of 5 mM into the HCCF where the reduction activity has been eliminated under the conditions described above in FIG. 13.

FIG. 15. The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 10 mM into the HCCF where the reduction activity has been eliminated due to the treatment described above in FIG. 13.

FIG. 16. Ocrelizumab Reduction: A digital gel-like image from Bioanalyzer analysis showing that ocrelizumab was reduced in an incubation experiment using a HCCF from a large scale manufacturing run (the "alpha" run).

FIG. 17. EDTA Inhibits Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the reduction of ocrelizumab was inhibited in an incubation experiment using a HCCF from the alpha run with EDTA added at a concentration of 20 mM to the HCCF whose reducing activity is demonstrated in FIG. 16.

US 8,574,869 B2

7

FIG. 18. The Lost Reduction Activity in "Beta Run" HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA: The reduction of ocrelizumab was observed in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 5 mM and 20 mM EDTA into the HCCF whose reduction activity had been lost (see FIG. 13). In contrast to the results shown in FIG. 17, the presence of EDTA did not block the reduction of ocrelizumab.

FIG. 19. Inhibition of Ocrelizumab Reduction: by (i) addition of EDTA, (ii) addition of CuSO_4 , or (iii) adjustment of pH to 5.5. All three different methods, (1) addition of EDTA, (2) addition of CuSO_4 , and (3) adjustment of pH to 5.5, used independently, were effective in inhibiting ocrelizumab reduction. This was demonstrated by the depicted quantitative Bioanalyzer results that showed that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was completely reduced in the control HCCF after 20 hours of HCCF hold time.

FIG. 20. Inhibition of Ocrelizumab Reduction by Air Sparging: Sparging the HCCF with air was effective in inhibiting ocrelizumab disulfide bond reduction. This was demonstrated by the quantitative Bioanalyzer results showing that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was almost completely reduced in the control HCCF after 5 hours of sparging with nitrogen.

FIG. 21 shows the V_L (SEQ ID NO. 24) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. 22 shows the V_H (SEQ ID No. 25) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. 23 is a schematic showing some steps of a typical large scale manufacturing process.

FIG. 24 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate.

FIG. 25 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 1 mM histidine sulfate+1 mM ATG.

FIG. 26 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.6 μM ATG (6:1 ATG:TrxR).

FIG. 27 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.4 μM ATG (4:1 ATG:TrxR).

FIG. 28 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.2 μM ATG (2:1 ATG:TrxR).

FIG. 29 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.1 mM autothiomalate (ATM).

FIG. 30 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.01 mM autothiomalate (ATM).

FIG. 31 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+20 μM CuSO_4 (4:1 Cu^{2+} :Trx).

FIG. 32 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1

8

μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+10 μM CuSO_4 (2:1 Cu^{2+} :Trx).

FIG. 33 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+5 μM CuSO_4 (1:1 Cu^{2+} :Trx).

FIG. 34 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+532 μM cystamine (20:1 cystamine:2H7 disulfide).

FIG. 35 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+266 μM cystamine (10:1 cystamine:2H7 disulfide).

FIG. 36 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+133 μM cystamine (5:1 cystamine:2H7 disulfide).

FIG. 37 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+26.6 μM cystamine (1:1 cystamine:2H7 disulfide).

FIG. 38 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate (pH=7.6)+2.6 mM cystine.

FIG. 39 is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+2.6 mM GSSG (oxidized glutathione).

FIG. 40 Reconstructed enzymatic reduction system. 1 mg/ml 2H7 (Variant A)+10 $\mu\text{g}/\text{mL}$ hexokinase, 50 $\mu\text{g}/\text{mL}$ glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thioredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+} , and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

FIG. 41 The thioredoxin system requires NADPH. 1 mg/ml 2H7 (Variant A)+5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In the present invention, in the context of proteins, including antibodies, in general, or with regard to any specific protein or antibody, the term "reduction" is used to refer to the reduction of one or more disulfide bonds of the protein or antibody. Thus, for example, the terms "ocrelizumab reduction" is used interchangeably with the term "ocrelizumab disulfide bond reduction" and the term "antibody (Ab) reduction" is used interchangeably with the term "antibody (Ab) disulfide bond reduction."

The terms "reduction" or "disulfide bond reduction" are used in the broadest sense, and include complete and partial reduction and reduction of some or all of the disulfide bonds, interchain or intrachain, present in a protein such as an antibody.

By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed

US 8,574,869 B2

9

within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term “therapeutic protein” or “therapeutic polypeptide” refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. “Manufacturing scale” production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

The term “therapeutic antibody” refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell’s death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A “biologically functional fragment” of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The term “diagnostic protein” refers to a protein that is used in the diagnosis of a disease.

The term “diagnostic antibody” refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A “biologically functional fragment” of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

“Purified” means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained.

The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An “essentially pure” protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An “essentially homogeneous” protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

10

As noted above, in certain embodiments, the protein is an antibody. “Antibodies” (Abs) and “immunoglobulins” (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term “antibody” is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv).

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier “monoclonal” indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature*, 256: 495 (1975); Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature*, 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101 (34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes

US 8,574,869 B2

11

encoding human immunoglobulin sequences (see, e.g., WO98/24893; WO96/34096; WO96/33735; WO91/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; Marks et al., *BioTechnology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996) and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurler and Gross, *Curr. Op. Biotech.* 5:428-433 (1994). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have

12

nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., *Bio/Technology* 10:779-783 (1992) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., *Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier et al., *Gene* 169:147-155 (1995); Yelton et al., *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as " V_H ." The variable domain of the light chain may be referred to as " V_L ." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called a, d, e, g, and m, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., *Cellular and Mol. Immunology*, 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion retains at least one, and as many

US 8,574,869 B2

13

as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for

14

example, EP 404,097; WO93/1161; Hudson et al., (2003) *Nat. Med.* 9:129-134; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) *Nat. Med.* 9:129-134.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150, 95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α -1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIc, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor- α and - β ; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1- α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally asso-

ciated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The terms “thioredoxin inhibitor” and “Trx inhibitor” are used interchangeably, and include all agents and measures effective in inhibiting thioredoxin activity. Thus, thioredoxin (Trx) inhibitors include all agents and measures blocking any component of the Trx, G6PD and/or hexokinase enzyme systems. In this context, “inhibition” includes complete elimination (blocking) and reduction of thioredoxin activity, and, consequently, complete or partial elimination of disulfide bond reduction in a protein, such as an antibody.

An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody’s natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms “Protein A” and “ProA” are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, GE Healthcare and Fermatech. Protein A is generally immobilized on a solid phase support material. The term “ProA” also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term “chromatography” refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term “affinity chromatography” and “protein affinity chromatography” are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or

proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms “non-affinity chromatography” and “non-affinity purification” refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A “cation exchange resin” refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from GE Healthcare) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from GE Healthcare). A “mixed mode ion exchange resin” refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J. T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term “anion exchange resin” is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (GE Healthcare).

A “buffer” is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The “loading buffer” is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

US 8,574,869 B2

17

The “intermediate buffer” is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term “wash buffer” when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The “elution buffer” is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A “regeneration buffer” may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term “substantially similar” or “substantially the same,” as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., K_d values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase “substantially reduced,” or “substantially different,” as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., K_d values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term “vector,” as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid,” which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as “recombinant expression vectors,” or simply, “expression vectors.” In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification,

18

“plasmid” and “vector” may be used interchangeably as the plasmid is the most commonly used form of vector.

“Percent (%) amino acid sequence identity” with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program

ALIGN-2 in that program’s alignment of A and B, and

where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

“Percent (%) nucleic acid sequence identity” is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter

sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

An "interfering RNA" or "small interfering RNA (siRNA)" is a double stranded RNA molecule less than about 30 nucleotides in length that reduces expression of a target gene. Interfering RNAs may be identified and synthesized using known methods (Shi Y., Trends in Genetics 19(1):9-12 (2003), WO/2003056012 and WO2003064621), and siRNA libraries are commercially available, for example from Dharmacon, Lafayette, Colo. Frequently, siRNAs can be successfully designed to target the 5' end of a gene.

II. Compositions and Methods of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); *PCR: A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells*, 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunocytochemistry in Practice*, 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); *Handbook of Experimental Immunology*, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A*

laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering*, 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology*.

1. Prevention of Disulfide Bond Reduction

The present invention concerns methods for the prevention of the reduction of disulfide bonds of proteins during recombinant production. In particular, the invention concerns methods for preventing the reduction of disulfide bonds of recombinant proteins during processing following fermentation. The methods of the invention are particularly valuable for large scale production of disulfide bond containing proteins, such as at a manufacturing scale. In one embodiment, the methods of the invention are useful for large scale protein production at a scale of greater than 5,000 L.

It has been experimentally found that disulfide bond reduction occurs during processing of the Harvested Cell Culture Fluid (HCCF) produced during manufacturing of recombinant proteins that contain disulfide bonds. Typically, this reduction is observed after cell lysis, especially mechanical cell lysis during harvest operations, when it reaches a certain threshold, such as, for example, from about 30% to about 70%, or from about 40% to about 60%, or from about 50% to about 60% total cell lysis. This threshold will vary, depending on the nature of the protein (e.g. antibody) produced, the recombinant host, the production system, production parameters used, and the like, and can be readily determined experimentally.

Theoretically, such reduction might result from a variety of factors and conditions during the manufacturing process, and might be caused by a variety of reducing agents. The present invention is based, at least in part, on the recognition that the root cause of this reduction is an active thioredoxin (Trx) or thioredoxin-like system in the HCCF.

The Trx enzyme system, composed of Trx, thioredoxin reductase (TrxR) and NADPH, is a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis. The results presented herein demonstrate that NADPH, which is required for activity of the Trx system is provided by glucose-6-phosphate dehydrogenase (G6PD) activity, which generates NADPH from glucose and ATP by hexokinase (see FIG. 4). These cellular enzymes (Trx system, G6PD, and hexokinase) along with their substrates are released into the CCF upon cell lysis, allowing reduction to occur. Accordingly, disulfide reduction can be prevented by inhibitors of the Trx enzyme system or upstream enzyme systems providing components for an active Trx system, such as G6PD and hexokinase activity.

For further details of these enzyme systems, or regarding other details of protein production, see, for example: Babson, A. L. and Babson, S. R. (1973) Kinetic Colorimetric Measurement of Serum Lactate Dehydrogenase Activity. *Clin. Chem.* 19: 766-769; Michael W. Laird et al., "Optimization of BLyS Production and Purification from *Escherichia coli*," *Protein Expression and Purification* 39:237-246 (2005); John C. Joly et al., "Overexpression of *Escherichia coli* Oxidoreductases Increases Recombinant Insulin-like Growth Factor-I Accumulation," *Proc. Natl. Acad. Sci. USA* 95:2773-2777 (March 1998); Dana C. Andersen et al., "Production Technologies for Monoclonal Antibodies and Their Fragments,"

Current Opinion in Biotechnology 15:456-462 (2004); Yariv Mazor et al., "Isolation of Engineered, Full-length Antibodies from Libraries Expressed in *Escherichia coli*," *Nature Biotech.* 25, 563-565 (1 Jun. 2007); Laura C. Simmons et al., "Expression of Full-length Immunoglobulins in *Escherichia coli*: Rapid and Efficient Production of Aglycosylated Antibodies," *Journal of Immunological Methods* 263:133-147 (2002); Paul H. Bessette et al., "Efficient Folding of Proteins with Multiple Disulfide Bonds in the *Escherichia coli* cytoplasm," *Proc. Natl. Acad. Sci.* 96(24):13703-08 (1999); Chaderjian, W. B., Chin, E. T., Harris, R. J., and Etcheverry, T. M., (2005) "Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed," *Biotechnol. Prog.* 21: 550-553; Gordon G., Mackow M. C., and Levy H. R., (1995) "On the mechanism of interaction of steroids with human glucose 6-phosphate dehydrogenase," *Arch. Biochem. Biophys.* 318: 25-29; Gromer S., Urig S., and Becker K., (2004) "The Trx System—From Science to Clinic," *Medicinal Research Reviews*, 24: 40-89; Hammes G. G. and Kochavi D., (1962a) "Studies of the Enzyme Hexokinase. I. Steady State Kinetics at pH 8," *J. Am. Chem. Soc.* 84:2069-2073; Hammes G. G. and Kochavi D., (1962b) "Studies of the Enzyme Hexokinase. III. The Role of the Metal Ion," *J. Am. Chem. Soc.* 84:2076-2079; Johansson C., Lillig C. H., and Holmgren A., (2004) "Human Mitochondrial Glutaredoxin Reduces S-Glutathionylated Proteins with High Affinity Accepting Electrons from Either Glutathione or Thioredoxin Reductase," *J. Biol. Chem.* 279:7537-7543; Legrand, C., Bour, J. M., Jacob, C., Capiaumont J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G., Demangel, C., Duval, D., and Hache J., (1992) "Lactate Dehydrogenase (LDH) Activity of the Number of Dead Cells in the Medium of Cultured Eukaryotic Cells as Marker," *J. Biotechnol.*, 25: 231-243; McDonald, M. R., (1955) "Yeast Hexokinase: ATP+ Hexose→Hexose-6-phosphate+ADP," *Methods in Enzymology*, 1: 269-276, Academic Press, NY; Sols, A., DelaFuente, G., Villar-Palasi, C., and Asensio, C., (1958) "Substrate Specificity and Some Other Properties of Bakers' Yeast Hexokinase," *Biochim Biophys Acta* 30: 92-101; Kirkpatrick D. L., Kuperus M., Dowdeswell M., Potier N., Donald L. J., Kunkel M., Berggren M., Angulo M., and Powis G., (1998) "Mechanisms of inhibition of the Trx growth factor system by antitumor 2-imidazolyl disulfides," *Biochem. Pharmacol.* 55: 987-994; Kirkpatrick D. L., Watson S., Kunkel M., Fletcher S., Ulhaq S., and Powis G., (1999) "Parallel syntheses of disulfide inhibitors of the Trx redox system as potential antitumor agents," *Anticancer Drug Des.* 14: 421-432; Milhausen, M., and Levy, H. R., (1975) "Evidence for an Essential Lysine in G6PD from *Leuconostoc mesenteroides*," *Eur. J. Biochem.* 50: 453-461; Pleasants, J. C., Guo, W., and Rabenstein, D. L., (1989) "A comparative study of the kinetics of selenol/diselenide and thiol/disulfide exchange reactions," *J. Am. Chem. Soc.* 111: 6553-6558; Whitesides, G. M., Lilburn, J. E., and Szajewski, R. P., (1977) "Rates of thiol-disulfide interchange reactions between mono- and dithiols and Ellman's reagent," *J. Org. Chem.* 42: 332-338; and Wipf P., Hopkins T. D., Jung J. K., Rodriguez S., Birmingham A., Southwick E. C., Lazo J. S., and Powis G., (2001) "New inhibitors of the Trx-TrxR system based on a naphthoquinone spiroketal natural product lead," *Bioorg. Med. Chem. Lett.* 11: 2637-2641.

According to one aspect of the present invention, disulfide bond reduction can be prevented by blocking any component of the Trx, G6PD and hexokinase enzyme systems. Inhibitors of these enzyme systems are collectively referred to herein as "thioredoxin inhibitors," or "Trx inhibitors." The Trx inhibi-

tors are typically added to the cell culture fluid (CCF), which contains the recombinant host cells and the culture media, and/or to the harvested cell culture fluid (HCCF), which is obtained after harvesting by centrifugation, filtration, or similar separation methods. The HCCF lacks intact host cells but typically contains host cell proteins and other contaminants, including DNA, which are removed in subsequent purification steps. Thus, the Trx inhibitors may be added before harvest and/or during harvest, preferably before harvest.

Alternatively or in addition other, non-specific methods can also be used to prevent the reduction of disulfide bond reduction following fermentation during the recombinant production of recombinant proteins, such as air sparging or pH adjustment. Certain reduction inhibition methods contemplated herein are listed in the following Table 1.

TABLE 1

Reduction Inhibition Methods	
Method ¹	Purpose
Addition of EDTA, EGTA, or citrate	To inhibit hexokinase
Addition of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, or lyxose	To inhibit hexokinase
Addition of epiandrosterone or dehydroepiandrosterone (DHEA)	To inhibit G6PD
Addition of pyridoxal 5'-phosphate or 1-fluoro-2,4-dinitrobenzene	To inhibit G6PD
Addition of metal ions such as Cu ²⁺ , Zn ²⁺ , Hg ²⁺ , Co ²⁺ , or Mn ²⁺	To inhibit Trx system
Addition of alkyl-2-imidazolyl disulfides and related compounds (e.g., 1-methylpropyl-2-imidazolyl disulfide ²) or naphthoquinone spiroketal derivatives (e.g., palmarumycin CP ₁) ²	To inhibit Trx
Addition of aurothioglucose (ATG) or aurothiomalate (ATM)	To inhibit TrxR
Air sparging	To deplete G6P and NADPH; oxidizing agent
Cystine	Oxidizing agent
Oxidized glutathione	Oxidizing agents
pH Adjustment to below 6.0	To reduce thiol-disulfide exchange rate and Trx system activity

¹Applied to CCF prior to harvest or in HCCF immediately after harvest.

²Currently not available commercially.

"Trx inhibitors" for use in the methods of the present invention include, without limitation, (1) direct inhibitors of Trx, such as alkyl-2-imidazolyl disulfides and related compounds (e.g., 1-methylpropyl-2-imidazolyl disulfide) (Kirkpatrick et al., 1998 and 1999, supra) and naphthoquinone spiroketal derivatives (e.g., palmarumycin CP₁) (Wipf et al., 2001, supra); (2) specific inhibitors of TrxR, including gold complexes, such as aurothioglucose (ATG) and aurothiomalate (ATM) (see, e.g., the review by Gromer et al., 2004), which are examples of irreversible inhibitors of TrxR; (3) metal ions, such as Hg²⁺, Cu²⁺, Zn²⁺, Co²⁺, and Mn²⁺, which can form readily complexes with thiols and selenols, and thus can be used in embodiments of the instant invention as inhibitors of TrxR or Trx; (4) inhibitors of G6PD, such as, for example, pyridoxal 5'-phosphate and 1-fluoro-2,4-dinitrobenzene (Milhausen and Levy 1975, supra), certain steroids, such as dehydroepiandrosterone (DHEA) and epiandrosterone (EA) (Gordon et al., 1995, supra); and (4) inhibitors of hexokinase activity (and thereby production of G6P for the G6PD), including chelators of metal ions, e.g. Mg²⁺, such as EDTA, and compounds that react with SH groups, sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose,

US 8,574,869 B2

23

2-C-hydroxy-methylglucose, xylose and lyxose (Sols et al., 1958, *supra*; McDonald, 1955, *supra*); further hexokinase inhibitors are disclosed in U.S. Pat. No. 5,854,067 entitled "Hexokinase Inhibitors." It will be understood that these inhibitors are listed for illustration only. Other Trx inhibitors exists and can be used, alone or in various combinations, in the methods of the present invention.

"Trx inhibitors" for use in the methods of the present invention also include reagents whereby the reduction of recombinantly produced antibodies or proteins may be reduced or prevented by decreasing the levels of enzymes of the Trx system, the pentose phosphate pathway or hexokinase at various points during the production campaign. In some embodiments, this reduction of enzyme levels may be accomplished by the use of targeted siRNAs, antisense nucleotides, or antibodies. To design targeted siRNAs or antisense nucleotides to the genes as found in CHO cells, these gene sequences are available from public databases to select sequences for targeting enzymes in different organisms. See Example 9 below for examples of the genes of the *E. coli* and mouse Trx system.

In addition to using inhibitors discussed above, it is also possible in certain embodiments of the instant invention to prevent the reduction of a recombinant protein to be purified by sparging the HCCF with air to maintain an oxidizing redox potential in the HCCF. This is a non-directed measure that can deplete glucose, G6P and NADPH by continuously oxidizing the reduced forms of Trx and TrxR. Air sparging of the HCCF tank can be performed, for example, with an air flow of about 100 liters to about 200 liters, such as, for example, 150 liters per minutes. Air sparging can be performed to reach an end-point percentage of saturation; for example, air sparging can be continued until the HCCF is about 100% saturated with air, or it can be continued until the HCCR is about 30% saturated with air, or until it is between about 100% saturated to about 30% saturated with air. The minimum amount of dissolved oxygen (dO_2) required for the desired inhibitory effect also depends on the antibody or other recombinant protein produced. Thus, for example, about 10% dO_2 (or about 10 scfm for continuous stream) will have the desired effect during the production of antibody 2H7 (Variant A), while Apomab might require a higher (about 30%) dO_2 .

In further embodiments of the instant invention, another non-directed method usable to block the reduction of the recombinant protein is lowering the pH of the HCCF. This embodiment takes advantage of particularly slow thiol-disulfide exchange at lower pH values (Whitesides et al., 1977, *supra*; Pleasants et al., 1989, *supra*). Therefore, the activity of the Trx system is significantly lower at pH values below 6, and thus the reduction of the recombinant protein, such as ocrelizumab, can be inhibited.

The non-directed approaches can also be combined with each other and/or with the use of one or more Trx inhibitors.

Disulfide bond reduction can be inhibited (i.e., partially or fully blocked) by using one or more Trx inhibitors and/or applying non-directed approaches following completion of the cell culture process, preferably to CCF prior to harvest or in the HCCF immediately after harvest. The optimal time and mode of application and effective amounts depend on the nature of the protein to be purified, the recombinant host cells, and the specific production method used. Determination of the optimal parameters is well within the skill of those of ordinary skill in the art.

For example, in a mammalian cell culture process, such as the CHO antibody production process described in the Examples herein, if cupric sulfate ($CuSO_4$ in the form of pentahydrate or the anhydrous form) is used as a Trx inhibitor,

24

it can be added to supplement the CCF or HCCF in the concentration range of from about 5 μM to about 100 μM , such as from about 10 μM to about 80 μM , preferably from about 15 μM to about 50 μM . Since some cell cultures already contain copper (e.g. about 0.04 μM $CuSO_4$ for the CHO cell cultures used in the Examples herein), this amount is in addition to the copper, if any, already present in the cell culture. Any copper (II) salt can be used instead of $CuSO_4$ as long as solubility is not an issue. For example, copper acetate and copper chloride, which are both soluble in water, can be used instead of $CuSO_4$. The minimum effective concentration may also depend on the antibody produced and the stage where the inhibitor is used. Thus, for example, when cupric sulfate is added pre-lysis, for antibody 2H7 (Variant A) the minimum effective concentration is about 30 μM , for Apomab is about 75 μM , and for antibody Variant C (see Table 2) is about 50 μM . When cupric sulfate is added in CC medium, for antibody 2H7 (Variant A) the minimum effective concentration is about 15 μM , for Apomab is about 25 μM , and for antibody Variant C is about 20 μM . One typical minimal $CuSO_4$ inhibitor concentration of 2 \times Trx concentration (or Trx equivalence).

EDTA can be used in a wide concentration range, depending on the extent of cell lysis, the recombinant host cell used, and other parameters of the production process. For example, when using CHO or other mammalian host cells, EDTA can be typically added in a concentration of between about 5 mM to about 60 mM, such as from about 10 mM to about 50 mM, or from about 20 mM to about 40 mM, depending on the extent of cell lysis. For lower degree of cell lysis, lower concentrations of EDTA will suffice, while for a cell lysis of about 75%-100%, the required EDTA concentration is higher, such as, for example, from about 20 mM to about 40 mM. The minimum effective concentration may also depend on the antibody produced. Thus, for example, for antibody 2H7 (Variant A) the minimum effective EDTA concentration is about 10 mM.

DHEA as a Trx inhibitor is typically effective at a lower concentration, such as for example, in the concentration range from about 0.05 mM to about 5 mM, preferably from about 0.1 mM to about 2.5 mM.

Other Trx inhibitors, such as aurothioglucose (ATG) and aurothiomalate (ATM) inhibit reduction of disulfide bonds in the μM concentration range. Thus, for example, ATG or ATM may be added in a concentration between about 0.1 mM to about 1 mM. While the minimum inhibitory concentration varies depending on the actual conditions, for ATG and ATM typically it is around 4 \times TrxR concentration.

It is noted that all inhibitors can be used in an excess amount, therefore, it is not always necessary to know the amount of Trx or TrxR in the system.

In a preferred embodiment, the mammalian host cell used in the manufacturing process is a chinese hamster ovary (CHO) cell (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)). Other mammalian host cells include, without limitation, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), Graham et al., *J. Gen. Virol.* 36:59 (1977); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary

US 8,574,869 B2

25

tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2); and myeloma or lymphoma cells (e.g. Y0, J558L, P3 and NS0 cells) (see U.S. Pat. No. 5,807,715).

A preferred host cell for the production of the polypeptides herein is the CHO cell line DP12 (CHO K1 dhfr⁻). This is one of the best known CHO cell lines, widely used in laboratory practice (see, for example, EP 0,307,247, published Mar. 15, 1989). In addition, other CHO-K1 (dhfr⁻) cell lines are known and can be used in the methods of the present invention.

The mammalian host cells used to produce peptides, polypeptides and proteins can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace (1979), Meth. in Enz. 58:44, Barnes and Sato (1980), Anal. Biochem. 102:255, U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985; or U.S. Pat. No. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GentamycinTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

A protocol for the production, recovery and purification of recombinant antibodies in mammalian, such as CHO, cells may include the following steps:

Cells may be cultured in a stirred tank bioreactor system and a fed batch culture, procedure is employed. In a preferred fed batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, a single step or multiple step culture

26

procedure may be employed. In a single step culture the host cells are inoculated into a culture environment and the processes are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture can be used.

5 In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

In certain embodiments, fed batch or continuous cell culture conditions may be devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO₂) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO₂) or a base (e.g., Na₂CO₃ or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30° C. to 38° C., and a suitable dO₂ is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

The cell culture environment during the production phase of the cell culture is typically controlled. Thus, if a glycoprotein is produced, factors affecting cell specific productivity of the mammalian host cell may be manipulated such that the desired sialic acid content is achieved in the resulting glycoprotein. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged. Further details of this process are found in U.S. Pat. No. 5,721,121, and Chaderjian et al., *Biotechnol. Prog.* 21(2):550-3 (2005), the entire disclosures of which are expressly incorporated by reference herein.

Following fermentation proteins are purified. Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins and components in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a

US 8,574,869 B2

27

physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through." Thus, purification of recombinant proteins from the cell culture of mammalian host cells may include one or more affinity (e.g. protein A) and/or ion exchange chromatographic steps.

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

For further details of the industrial purification of therapeutic antibodies see, for example, Fahrner et al., *Biotechnol. Genet. Eng. Rev.* 18:301-27 (2001), the entire disclosure of which is expressly incorporated by reference herein.

In addition to mammalian host cells, other eukaryotic organisms can be used as host cells for expression of the recombinant protein. For expression in yeast host cells, such as common baker's yeast or *Saccharomyces cerevisiae*, suitable vectors include episomally-replicating vectors based on the 2-micron plasmid, integration vectors, and yeast artificial chromosome (YAC) vectors. Other yeast suitable for recombinant production of heterologous proteins include *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290: 140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529; Fleer et al., *Bio/Technology*, 2: 968 975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., *J. Bacteriol.*, 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarius* (ATCC 36,906; Van den Berg et al., *Bio/Technology*, 8: 135 (1990)), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., *J. Basic Microbiol.*, 28: 265 278 (1988)); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case et al., *Proc. Natl. Acad. Sci. USA*, 76: 5259 5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 Oct. 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 Jan. 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., *Biochem. Biophys. Res. Commun.*, 112: 284 289 (1983); Tilburn et al., *Gene*, 26: 205 221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA*, 81: 1470 1474 (1984)) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4: 475 479 (1985)). Methylotrophic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, *Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, *The Biochemistry of Methylotrophs*, 269 (1982). Expression systems for the listed and other yeasts are well known in the art and/or are commercially available.

28

For expression in insect host cells, such as Sf9 cells, suitable vectors include baculoviral vectors. For expression in plant host cells, particularly dicotyledonous plant hosts, such as tobacco, suitable expression vectors include vectors derived from the Ti plasmid of *Agrobacterium tumefaciens*.

The methods of the present invention also extend to cultures of prokaryotic host cells. Prokaryotic host cells suitable for expressing antibodies and other proteins to be protected by means of the instant invention include Archaeobacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (e.g., *E. coli*), *Bacilli* (e.g., *B. subtilis*), *Enterobacteria*, *Pseudomonas* species (e.g., *P. aeruginosa*), *Salmonella typhimurium*, *Serratia marcescans*, *Klebsiella*, *Proteus*, *Shigella*, *Rhizobia*, *Vitreoscilla*, or *Paracoccus*. In one embodiment, gram-negative cells are used. Examples of *E. coli* strains include strain W3110 (Bachmann, *Cellular and Molecular Biology*, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 Δ fluA (Δ tonA) ptr3 lac Iq lacL8 Δ ompT Δ (nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308 (ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., *Proteins*, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, *Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Methods for the production, recovery and purification of recombinant proteins from non-mammalian host cell cultures are also well known in the art. If the polypeptide is produced in a non-mammalian cell, e.g., a microorganism such as fungi or *E. coli*, the polypeptide will be recovered inside the cell or in the periplasmic space (Kipriyanov and Little, *Molecular Biotechnology*, 12: 173 201 (1999); Skerra and Pluckthun, *Science*, 240: 1038 1040 (1988)). Hence, it is necessary to release the protein from the cells to the extracellular medium by extraction such as cell lysis. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration.

Cell lysis is typically accomplished using mechanical disruption techniques such as homogenization or head milling. While the protein of interest is generally effectively liberated, such techniques have several disadvantages (Engler, *Protein Purification Process Engineering*, Harrison eds., 37 55 (1994)). Temperature increases, which often occur during processing, may result in inactivation of the protein. Moreover, the resulting suspension contains a broad spectrum of contaminating proteins, nucleic acids, and polysaccharides. Nucleic acids and polysaccharides increase solution viscosity, potentially complicating subsequent processing by centrifugation, cross-flow filtration, or chromatography. Complex associations of these contaminants with the protein of interest can complicate the purification process and result in unacceptably low yields. Improved methods for purification of heterologous polypeptides from microbial fermentation

US 8,574,869 B2

29

broth or homogenate are described, for example, in U.S. Pat. No. 7,169,908, the entire disclosure of which is expressly incorporated herein by reference.

It is emphasized that the fermentation, recovery and purification methods described herein are only for illustration purposes. The methods of the present invention can be combined with any manufacturing process developed for the production, recovery and purification of recombinant proteins.

2. Antibodies

In a preferred embodiment, the methods of the present invention are used to prevent the reduction of inter- and/or intrachain disulfide bonds of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest*, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors*, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF- α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J. Immunol.* 156(4): 1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9): 1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_5\beta_1$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against Fc γ RI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23 Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Suppl): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis

30

et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl): 5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al., *Cancer Res* 55(23 Suppl): 5899s-5907s (1995)); anti-Ep-CAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti- $\alpha\beta_3$ antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2): 435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-16, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2): 435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application

US 8,574,869 B2

31

No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); WO 02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albiter et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegal); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821 (Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332,865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1)

32

30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): S197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002; Tuscan, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized 2H7 anti-CD20 antibodies. In specific embodiments, the humanized 2H7 antibody is an antibody listed in Table 2.

TABLE 2

Humanized anti-CD20 Antibody and Variants Thereof				
2H7 variant	V _L SEQ ID NO.	V _H SEQ ID NO.	Full L chain SEQ ID NO.	Full H chain SEQ ID NO.
A	1	2	6	7
B	1	2	6	8
C	3	4	9	10
D	3	4	9	11
F	3	4	9	12
G	3	4	9	13
H	3	5	9	14
I	1	2	6	15

Each of the antibody variants A, B and I of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 1)

DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHVYQKPGKAPKPLIYA

PSNLASGVPSRFSGSGSGTDFTLTISSLPEDFATYYCQQWSFNPTTFG

QGTEVEIKR;
andthe heavy chain variable sequence (V_H):

(SEQ ID NO: 2)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA

IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARV

YYSNSYWFYFDVWGQGLTVTVSS.

Each of the antibody variants C, D, F and G of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 3)

DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQKPGKAPKPLIYAP

SNLASGVPSRFSGSGSGTDFTLTISSLPEDFATYYCQQWAFNPTTFGQG

TKVEIKR,
and

(part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" *New Scientist* (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): 5370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.*

US 8,574,869 B2

33

the heavy chain variable sequence (V_H):

(SEQ ID NO: 4)
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSASYWYFDVWGQGTLLVTVSS.

The antibody variant H of Table 2 comprises the light chain variable sequence (V_L) of SEQ ID NO:3 (above) and the heavy chain variable sequence (V_H):

(SEQ ID NO: 5)
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSYRYWYFDVWGQGTLLVTVSS

Each of the antibody variants A, B and I of Table 2 comprises the full length light chain sequence:

(SEQ ID NO: 6)
 DIQMTQSPSSLSASVGDRTITCRASSSVSYMHYQQKPGKAPKPLIYAP
 SNLASGVPSRFSGSGSDFTLTISLQPEDFATYYCQQWFSNPPTFGQG
 TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFPYREAKVQWKVD
 NALQSGNSQESVTEQDSKSTYLSLSTLTLSKADYEKHKVYACEVTHQGL
 SSPVTKSFNRGEC.

Variant A of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 7)
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSNSYWYFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSP
 GK.

Variant B of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 8)
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSNSYWYFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ

34

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YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSP
 GK.

Variant I of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 15)
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSNSYWYFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSP
 GK.

Each of the antibody variants C, D, F, G and H of Table 2 comprises the full length light chain sequence:

(SEQ ID NO: 9)
 DIQMTQSPSSLSASVGDRTITCRASSSVSYLHWYQQKPGKAPKPLIYAP
 SNLASGVPSRFSGSGSDFTLTISLQPEDFATYYCQQWAFNPPTFGQG
 TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFPYREAKVQWKVD
 NALQSGNSQESVTEQDSKSTYLSLSTLTLSKADYEKHKVYACEVTHQGL
 SSPVTKSFNRGEC.

Variant C of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 10)
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSASYWYFDVWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSP
 GK.

US 8,574,869 B2

35

Variant D of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 11) 5
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSASYWYFDVWGQGTLTVTSSASTKGPSVPFLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTT
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
 GK.

Variant F of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 12) 30
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSASYWYFDVWGQGTLTVTSSASTKGPSVPFLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVSVLTVLHQDWLNGKEYKCKVSNALPAPIAATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTT
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
 GK.

Variant G of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 13) 50
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSASYWYFDVWGQGTLTVTSSASTKGPSVPFLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVSVLTVLHQDWLNGKEYKCKVSNALPAPIAATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTT
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHWHYTQKSLSLSP
 GK.

36

Variant H of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 14) 5
 EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKLEWVGA
 IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
 YYSYRYWYFDVWGQGTLTVTSSASTKGPSVPFLAPSSKSTSGGTAALGCL
 VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGT
 QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
 KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
 YNATYRVVSVLTVLHQDWLNGKEYKCKVSNALPAPIAATISKAKGQPRE
 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTT
 PVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP
 GK.

In certain embodiments, the humanized 2H7 antibody of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized 2H7 antibody compositions of the present invention include compositions of any of the preceding humanized 2H7 antibodies having a Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to FcγRIIIA(F158), which is not as effective as FcγRIIIA (V158) in interacting with human IgG. FcγRIIIA (F158) is more common than FcγRIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized 2H7 antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized 2H7 in antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the second antigen is selected from the group consisting of CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

US 8,574,869 B2

37

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors over-express the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In one embodiment, the anti-HER2 antibody comprises the following V_L and V_H domain sequences:

humanized 2C4 version 574 antibody V_L
(SEQ ID NO: 16)
DIQMTQSPSSLSASVGDRVTITCR**ASQDVSIGVA**WYQQKPGKAPKLLIYS
ASRYRTGVPSTRFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPY**TFGQ
GTKVEIK.
and humanized 2C4 version 574 antibody V_H
(SEQ ID NO: 17)
EVQLVESGGGLVQPGGSLRLSCAAS**GF**T**FTD**Y**TMD**WVRQAPGKGLEWVAD
VNPNSGGS**IYNQ**R**FK**GRFTLSVDRSKNTLYLQMN**SLRAEDTAVYYCARNL**
GPSFYFDYWGQGLTVTVSS.

In another embodiment, the anti-HER2 antibody comprises the V_L (SEQ ID NO:18) and V_H (SEQ ID NO:19) domain sequences of trastuzumab as shown in FIG. 21 and FIG. 22, respectively.

Other HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS (USA)* 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

The anti-VEGF antibodies may, for example, comprise the following sequences:

In one embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:20):

DIQMTQTSS LSASLGDRVI ISCSASQDIS NYLNWYQQKP
DGTVKVLIYF TSSLHSGVPS RFGSGSGSTD YSLTISNLEP
EDIATYYCQQ YSTVPWTFGG GTKLEIKR;
and

38

the following V_H sequence (SEQ ID NO:21):

5 EIQLVQSGPE LKQPGETVRI SCKASGYTFT NYGMNWVKQA
PGKGLKWMGW INTYTGEPTY AADFKRRFTF SLETSASTAY
LQISNLKND TATYFCAKYP HYYGSSHWYF DVWGAGTTVT VSS.

10 In another embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:22):

15 DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP
GKAPKVLIIYF TSSLHSGVPS RFGSGSGSTD FTLTISSLQP
EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

20 the following V_H sequence (SEQ ID NO:23):

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA
PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
25 LQMN**SLRAEDTAVYYCAKYP** HYYGSSHWYF DVWGQGLTVT VSS.

In a third embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:24):

30 DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP
GKAPKVLIIYF TSSLHSGVPS RFGSGSGSTD FTLTISSLQP
EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:25):

40 EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
LQMN**SLRAEDTAVYYCAKYP** YYYGTSHWYF DVWGQGLTVT VSS.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva® (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for an anti-human CD11a antibody comprising the V_L and V_H sequences of HuMHM24 below:

55 V_L (SEQ ID NO: 26):
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLA**WYQQKPGKAPKLLIYS**
GSTLQSGVPSRFGSGSGTDFTLTIS**SLQPEDFATYYCQ**HNEYPLTFGQ
GTKVEIKR;
60 and
 V_H (SEQ ID NO: 27):
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM
IHPDSETRYNQKFDRFTISVDKSKNTLYLQMN**SLRAEDTAVYYCARGI**
65 YFYGTTFDYWGQGLTVTVSS.

US 8,574,869 B2

39

The anti-human CD11a antibody may comprise the V_H of SEQ ID NO:27 and the full length L chain of HuMHM24 having the sequence of:

(SEQ ID NO: 28)
 DIQMTQSPSSLSASVGRVTITCRASKTISKYLAWYQQKPKAPKLLIYS
 GSTLQSGVPSRFSGSGSDFTLTITSLQPEDFATYYCQQHNEYPLTFGQ
 GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
 DNALQSGNSQESVTEQDSKSDSTYSLSTLTLSKADYEKHKVYACEVTHQG
 LSSPVTKSFNRGEC,
 or

the L chain above with the H chain having the sequence of:

(SEQ ID NO: 29)
 EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWRQAPGKGLEWVGM
 IHPSDSETRYNQKFKDRFTTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI
 YFYGTTYFDYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV
 KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ
 TYICNVNHPKSNKTKVDKKVEPKSKDKTHTCPPCPAPELLGSPSVFLFPPK
 PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
 NSTYRVVSVLTVLHQDWLNGKEYCKVSNKALPAPIEKTISKAKGQPREP
 QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
 VLDSGDSFPLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPG
 K.

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference.

3. Other Disulfide-Containing Proteins

In addition to antibodies, the methods of the present invention find utility in the manufacturing of other polypeptides including disulfide bonds. Representative examples of such polypeptides include, without limitation, the following therapeutic proteins: tissue plasminogen activators (t-PAs), such as human tissue plasminogen activator (htPA, alteplase, ACTIVASE®), a thrombolytic agent for the treatment of myocardial infarction; a TNKase™, a ht-PA variant with extended half-life and fibrin specificity for single-bolus administration; recombinant human growth hormone (rhGH, somatropin, NUTROPIN®, PROTROPIN®) for the treatment of growth hormone deficiency in children and adults; and recombinant human deoxyribonuclease I (DNase I) for the treatment of cystic fibrosis (CF).

Examples of disulfide-containing biologically important proteins include growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors

40

such as factor VIIIc, factor IX, tissue factor, and von Willibrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

4. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically identified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150, 95, VLA-4, ICAM-1, VCAM and αv/133 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group

US 8,574,869 B2

41

antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with $\frac{1}{5}$ to $\frac{1}{10}$ the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxan-

42

thine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

US 8,574,869 B2

43

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

44

In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immunol.*, 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first

antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab' -TNB derivatives is then reconverted to the Fab' -thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab' -TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab' -SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by

the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H - C_H1 - V_H - C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1 , hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L - AC_L ;
 AC_H -(AC_H , AC_L - AC_H , AC_L - V_H - C_H , or V_L - C_L - AC_H);
 AC_L - AC_H -(AC_L - AC_H , AC_L - V_H - C_H , V_L - C_L - AC_H , or V_L - C_L - V_H - C_H);
 AC_L - V_H - C_H -(AC_H , or AC_L - V_H - C_H , or V_L - C_L - AC_H);
 V_L - C_L - AC_H -(AC_L - V_H - C_H , or V_L - C_L - AC_H); and
 $(A-Y)_n$ -(V_L - C_L - V_H - C_H)₂,
 wherein each A represents identical or different adhesin amino acid sequences;
 V_L is an immunoglobulin light chain variable domain;
 V_H is an immunoglobulin heavy chain variable domain;

US 8,574,869 B2

47

C_L is an immunoglobulin light chain constant domain;
 C_H is an immunoglobulin heavy chain constant domain;
 n is an integer greater than 1;
 Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_{H2} domain, or between the C_{H2} and C_{H3} domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Va.

48

Example 1

Description of Materials and Methods

The following materials and methods were used in Examples 2-8 below.

Materials

Materials and devices used in the experiments described in the experimental examples include: stainless steel vials (mini-tanks, Flow Components, Dublin, Calif.; short (50 cc) and tall (55 cc)); dialysis tubing (Spectra/Por, 6-8000 MWCO, cat. #132645), 0.22 μ m filter (Millipore Millipak Gamma Gold cat. # MPGL04GH2); phosphate buffered saline (PBS, EMD, cat. #6506); ethylenediaminetetraacetic acid (EDTA, Sigma, cat. # E4884); α -nicotinamide adenine dinucleotide phosphate (NADPH, Calbiochem, cat. #481973); dehydroepiandrosterone (DHEA, TCI, cat. # D0044); cupric sulfate (Sigma, cat. # C8027), glucose-6-phosphate (G6P, Calbiochem, cat. #346764); aurothioglucose (ATG, USP, cat. #1045508); aurothiomalate (ATM, Alfa Aesar, cat. #39740); reduced glutathione (GSH, J. T. Baker, cat. # M770-01); monobromobimane (mBB, Fluka, cat. #69898); histidine (J. T. Baker, cat. #2080-05); sodium sulfate (J. T. Baker, cat. #3897-05); Trx (Sigma, cat. # T8690); TrxR (Sigma, cat. # T9698). All chemicals and reagents were used as received with no further purification. Stock solutions of EDTA (250 mM, pH 7.5), CuSO_4 (10 mM), ATG (30 mM), ATM (30 mM), NADPH (75 mM), G6P (300 mM) were prepared for use in the mini-tank time course studies.

Generation of Cell Culture Fluid (CCF)

In order to generate ocrelizumab CCF for the various reduction studies, a representative small-scale fermentation process was utilized similar to the methods described previously (Chaderjian et al., 2005). Briefly, 3 liter glass stirred-tank Applikon® bioreactors fitted with pitched blade impellers were used for the inoculum-train and production cultures with the ocrelizumab media components. The bioreactors were outfitted with calibrated dissolved oxygen (DO), pH and temperature probes. DO, pH, temperature, and agitation rate were controlled using digital control units to the defined parameters of the ocrelizumab manufacturing process. The working volume for both the inoculum-train and production cultures was 1.5 L. Daily samples were analyzed on a NOVA Bioprofile blood gas analyzer to ensure the accuracy of the on-line value for pH and dissolved oxygen as well as to monitor the glucose, lactate, ammonium, glutamine, glutamate, and sodium concentrations in the cultures. Daily samples were also taken to monitor cell growth, viability, and titer. Cell growth was measured both by viable cell counts using a ViCell as well as on a packed cell volume (PCV) basis. Culture viability was determined by trypan blue exclusion on a ViCell instrument. Supernatant samples were assayed by an HPLC-based method to measure ocrelizumab titer values.

Harvested Cell Culture Fluid (HCCF) Preparation

Complete lysis of CCF was achieved by high pressure homogenization using a Microfluidics HC-8000 homogenizer. The pressure regulator of the instrument was set to 4,000-8,000 psi, and the CCF was pulled in through the homogenizer to obtain complete cell lysis (membrane breakage) after a single pass. The CCF homogenate was collected once water was purged through the system. The homogenate was transferred to centrifuge bottles and centrifuged in a Sorval RC-3B rotor centrifuge at 4,500 rpm for 30 minutes at 20° C. The centrate was decanted and then depth filtered followed by 0.22 μ m sterile filtration using a peristaltic pump with silicon tubing to generate the final HCCF from the homogenized CCF (100% cell lysis). Alternatively, the CCF

was centrifuged straight from the fermentor without any homogenization and then the centrate was filtered with a sterile 0.22 μ m filter to generate the HCCF.

Mini-Tank Handling

A laminar flow hood was used in handling all mini-tanks and all materials used in the HCCF incubation experiments were either autoclaved or rinsed using 70% isopropanol to minimize bacterial contamination.

Lactate Dehydrogenase Assay

For lactate dehydrogenase assay, see Babson & Babson (1973) and Legrand et al., (1992), which are hereby incorporated by reference.

Dialysis Experiment

A dialysis experiment was carried out in order to determine whether the components causing reduction of ocrelizumab were small molecules or macromolecules (i.e. enzymes). A sample of 3 mL of purified and formulated ocrelizumab (30.2 mg/mL) was dialyzed against 1 L of phosphate buffered saline (PBS, 10 mM pH 7.2) for 24 hours and the PBS was changed after 8 hours. The concentration of the ocrelizumab sample was then adjusted to 1 mg/mL using the absorbance at 280 nm. Aliquots were stored at -70° C. prior to use. Dialysis tubing was hydrated overnight in a 0.05% azide solution and rinsed with sterile water prior to use. The HCCF obtained from homogenization of CCF from a 3-L fermentor was thawed and filtered through a 0.22 μ m Millipak filter using a peristaltic pump. Six short mini-tanks were filled with 30 mL of HCCF each. To each mini-tank, 500 μ L of ocrelizumab sample in sealed dialysis tubing was added. The mini-tanks were sealed and loaded into a bench top mixer (Barnstead Lab-Line MAX Q 4000) operating at 35 rpm and ambient temperature. For each time-point, one mini-tank was removed from the mixer, and aliquots of the HCCF (in the mini-tank) and ocrelizumab sample (in the dialysis bag) were taken and stored at -70° C. until analyzed with the free thiol assay and the Bioanalyzer assay (described below).

Test Inhibitors for Reduction in a Small-Scale In Vitro System

A tall mini-tank was filled with 27 mL of HCCF. Depending on the experiment design, various reagents (NADPH, G6P, inhibitors of G6PD or TrxR) were added to the desired concentration, and the final volume in the mini-tank was brought to 30 mL with PBS (10 mM pH 7.2). The mini-tanks were sealed and loaded into a bench top mixer running at 35 rpm and ambient temperature. At each-time point for sampling, the exteriors of the mini-tanks were sterilized with 70% IPA and opened in a laminar flow hood for the removal of an aliquot. The mini-tanks were then re-sealed and loaded back into the bench top mixer. All aliquots were stored at -70° C. until analyzed with the free thiol assay and Bioanalyzer assay (described below).

In Vitro Trx/TrxR reductase Studies

A commercial TrxR (rat liver) solution (4 μ M) was diluted with water to yield a 2.86 μ M solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μ M solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μ L PBS, 25 μ L NADPH, 16 μ L formulated ocrelizumab solution (30.2 mg/mL) and 5 μ L Trx were gently mixed. The reaction was initiated by the addition of 17.5 μ L TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μ L were taken at each sampling time-point and stored at -70° C. until analyzed by the Bioanalyzer assay (see below). Controls were performed to determine if the enzymatic pathway was active when an enzyme was omitted by substituting an equal volume of PBS for either Trx and/or TrxR in the reaction mixture.

matic pathway was active when an enzyme was omitted by substituting an equal volume of PBS for either Trx and/or TrxR in the reaction mixture.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of 5 μ L ATG or ATM. To demonstrate the inhibition of Trx system by Cu^{2+} , 2.5 μ L of CuSO_4 (10 mM) was added to reaction mixture using the same enzymes but a different buffer (10 mM histidine, 10 mM Na_2SO_4 , 137 mM NaCl, 2.5 mM KCl, pH 7.0) to prevent formation of insoluble $\text{Cu}_3(\text{PO}_4)_2$.

Free Thiol Assay

A standard curve using GSH was generated in PBS (10 mM, pH 6.0 \pm 0.05). From a 110 mM GSH solution, standards were prepared at concentrations of 0, 5.5, 11, 22, 44, 55, 110 and 550 μ M through serial dilution. From an acetonitrile stock solution of mBB (10 mM stored at -20° C.), a 100 μ M solution of mBB was prepared in PBS (10 mM, pH 10.0 \pm 0.05) and stored away from light.

In a black, flat bottomed 96 well plate, 100 μ L of mBB was dispensed into each well. For the standard curve, 10 μ L of standard GSH solution was added yielding a working pH of 8.0 \pm 0.2. For samples, 10 μ L of sample was added to the wells. All wells were prepared in triplicate. The plate was incubated at room temperature for 1 hour in the dark then read using a fluorescence plate reader (Molecular Devices SpectraMax® Gemini XS) with an excitation wavelength of 390 nm and an emission wavelength of 490 nm. A linear standard curve was generated using the average result of the three standard wells plotted versus GSH concentration. Free thiol levels in samples were calculated from the linear equation of the standard curve using the average value of the three sample wells.

Bioanalyzer Assay

Capillary electrophoresis measurements were acquired using the Agilent 2100 Bioanalyzer. Sample preparation was carried out as described in the Agilent Protein 230 Assay Protocol (manual part number G2938-90052) with minor changes. HCCF samples were diluted, 1:4 and Protein A samples were diluted to 1.0 g/L with water prior to preparation. For HCCF samples at the denaturing step, 24 μ L of a 50 mM iodoacetamide (IAM), 0.5% SDS solution was added in addition to the 2 μ L of denaturing solution provided. For Protein A samples, 0.5% SDS with no IAM and 2 μ L of denaturing solution were used. Digital gel-like images were generated using Agilent 2100 Expert software.

Stock Solutions for HCCF Hold Time Studies

Three separate stock solutions were used in the lab scale HCCF hold time studies: (1) 250 mM stock solution of EDTA (pH 7.4) prepared using EDTA, disodium dihydrate (Mallinckrodt, cat. #7727-06 or Sigma, cat. # E-5134) and EDTA, tetrasodium dihydrate (Sigma, cat. #E-6511), (2) 50 mM stock solution of cupric sulfate pentahydrate (CuSO_4 , Sigma, cat. # C-8027), and (3) 1 M acetic acid solution (Mallinckrodt, cat. # V193).

Inhibitor Additions and Cell Culture Fluid (CCF) Blending

A stock solution of either 250 mM EDTA or 50 mM CuSO_4 was added to the CCF prior to homogenization to evaluate a range of final concentrations to prevent antibody disulfide reduction. Once the final HCCF was generated from the homogenized CCF, these solutions were then mixed with the HCCF generated from the non-homogenized CCF (also containing EDTA or CuSO_4) in order to dilute and decrease the total level of cell lysis to below the 100% maximum. Alternatively, a stock solution of 1 M acetic acid was added to a final blended HCCF solution (homogenized CCF and non-homogenized CCF) to decrease the pH of the solution to prevent antibody disulfide reduction.

US 8,574,869 B2

51

Approximately 30-50 mL of each HCCF solution (containing EDTA, CuSO₄, acetic acid, or no addition for the control) was held in a 50 mL 316L stainless steel vial. The vial was sealed with a clamp, and the solution was not aerated or agitated. The vial was stored at room temperature (18-22° C.).

Similar results can be obtained with other oxidizing agents, such as, for example, cystine and oxidized glutathione.

Air Sparging

To evaluate air sparging of the HCCF generated from homogenized CCF to prevent antibody disulfide reduction, 3-L glass or 15-L stainless steel vessels were utilized. Approximately 1-5 L of HCCF was 0.22 µm sterile filtered into each sterilized vessel. Experimental conditions were maintained at 18-22° C. and 50 (15-L fermentor) or 275 rpm (3-L fermentor) agitation either with or without pH control by the addition of carbon dioxide. Solutions were either sparged with air to increase the dissolved oxygen level to air saturation or with nitrogen (control) to remove any dissolved oxygen in solution. Gas flow to each vessel was variable dependent upon whether a constant aeration rate was used or a minimum level of dissolved oxygen was maintained. At pre-determined time points, 25-50 mL samples were removed from both vessels and purified over a lab scale protein A affinity resin prior to analysis.

Protein A Processing

Antibody in harvested cell culture fluid samples can be captured and purified using a specific affinity chromatography resin. Protein A resin (Millipore, Prosep-vA High Capacity) was selected as the affinity resin for antibody purification. The resin was packed in a 0.66 cm inner diameter glass column (Omnifit®) with a 14 cm bed height resulting in a 4.8 mL final column volume. Chromatography was performed using an AKTA Explorer 100 chromatography system (GE Healthcare).

The resin was exposed to buffers and HCCF at a linear flow rate between 350-560 cm/hr. The resin was equilibrated with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1. For each purification, the resin was loaded between 5-15 mg antibody per mL of resin. The antibody concentration in the HCCF was determined using an immobilized protein A HPLC column (Applied Biosystems, POROS A). After loading, the resin was washed with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M TMAC, pH 7.1, and then the antibody was eluted using 0.1M acetic acid, pH 2.9. Elution pooling was based on UV absorbance at 280 nm measured inline after the column. The purified elution pools were pH-adjusted using 1 M Sodium HEPES to pH 5.0-5.5. After regeneration of the resin with 0.1M phosphoric acid, the same or similar packed resins were used for subsequent purification of other HCCF solutions.

The antibody concentration in the purified protein A pool was measured using UV spectrometry at 280 nm. The purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa molecular weight.

Example 2

Dialysis Experiment

A dialysis experiment was designed and carried out to determine if the reduction of ocrelizumab was caused by small reducing molecules or macromolecules (e.g., enzymes). In this dialysis experiment, purified intact ocrelizumab was placed in a dialysis bag with a molecular weight cut off (MWCO) of 7000 and incubated the dialysis bag in

52

HCCF containing ocrelizumab in a stainless steel mini-tank. As shown in FIGS. 1 and 2, the ocrelizumab inside the bag was not reduced after the incubation period (FIG. 1), whereas the ocrelizumab outside the bag in the HCCF was significantly reduced soon after the incubation started. This was evidenced by the loss of intact ocrelizumab (~150 kDa) and the formation of ocrelizumab fragments (various combinations of heavy and light chains) (FIG. 2). The mass spectrometry analysis of the ocrelizumab in the protein A elution pools from the reduced manufacturing runs indicated that those observed fragments were formed by reduction of only the inter-chain disulfide bonds.

The free thiol measurement showed that no free thiols were present inside the dialysis bag at the beginning of the incubation; however the levels of free thiols inside and outside the dialysis bag become comparable in less than five hours after the incubation started, indicating that the small molecule components in the HCCF are fully equilibrated inside and outside the dialysis bag (FIG. 3). Since the reduction was observed only outside but not inside the dialysis bag with a MWCO of 7000 Da, the molecular weight of the reducing molecule(s) must be greater than 7000 Da. Thus, an enzymatic reaction is responsible for the reduction of ocrelizumab.

Example 3

Reduction of Ocrelizumab (rhuMAb 2H7, Variant A) by Trx/TrxR In Vitro

The Trx system was tested for its ability to reduce ocrelizumab in vitro by incubating intact ocrelizumab with Trx, TrxR, and NADPH. The Bioanalyzer results indicate that ocrelizumab was reduced in vitro by the Trx system (FIG. 5). The rate of reduction in this in vitro system appears to be slower than that in the HCCF (for example when compared to the reduction shown in FIG. 2). This is likely due to lower concentrations of the enzymes (Trx and Trx-R) and/or the buffer system used in the in vitro reaction because reaction rate of Trx system is dependent on both the enzyme concentrations and buffer systems.

Example 4

Inhibitors of the Trx System

(i) Inhibition of Reduction of Recombinant Antibody by Cupric Sulfate

Cupric sulfate is known for its ability to provide oxidizing redox potential and has been used in the cell culture processes to minimize free thiol (i.e., minimize unpaired cysteine) levels in recombinant antibody molecules (Chaderjian et al., 2005, supra). Cupric sulfate was tested for efficacy in inhibiting the Trx system in vitro and the subsequent reduction of ocrelizumab. In this in vitro reduction experiment, the buffer system was changed from PBS to histidine sulfate to avoid the formation of insoluble Cu₃(PO₄)₂. FIG. 8 shows that ocrelizumab was readily reduced by the Trx system in the histidine sulfate buffer (even faster than in PBS buffer). The addition of CuSO₄ to this reaction clearly inhibits the ocrelizumab reduction (FIG. 9).

(ii) Inhibition of Reduction of Recombinant Antibody in HCCF by ATG and ATM

Two commercially available specific inhibitors of TrxR, aurothioglucose (ATG) and aurothiomalate (ATM), were tested for their ability to inhibit the Trx system in vitro and the reduction of ocrelizumab. Both ATG and ATM can effectively

inhibit the reduction of ocrelizumab in the assay described above (see FIGS. 6 and 7). The addition of aurothioglucose or aurothiomalate, at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. 5 effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis.

If the Trx system was active in the HCCF and reduced ocrelizumab as observed in the manufacturing runs resulting in reduced antibody molecules or in the lab scale experiments, both gold compounds (ATG and ATM) should be able to inhibit the reduction of ocrelizumab in HCCF. FIG. 10 shows that ocrelizumab was readily reduced in an HCCF from homogenized CCT generated from a 3-L fermentor after a period of incubation. However, the ocrelizumab reduction event was completely inhibited when either 1 mM ATG or ATM was added to the HCCF (FIGS. 11 and 12). These results demonstrated that the Trx system is active in the HCCF and is directly responsible for the reduction of ocrelizumab.

Example 5

The Source of NADPH for Trx System Activity and the Roles of G6P and Glucose in Reduction Mechanism

The reduction of disulfides by the Trx system requires the reducing equivalents from NADPH (FIG. 4). The main cellular metabolic pathway that provides NADPH for all reductive biosynthesis reactions is the pentose phosphate pathway. For the antibody reduction event to occur, the enzymes in this pathway must be still active in the HCCF in order to keep the Trx system active. At a minimum, the first step in the pentose phosphate pathway (catalyzed by G6PD) must be active to reduce NADP⁺ to NADPH while converting G6P to 6-phosphogluconolactone. In addition, G6P is most likely produced from glucose and adenosine 5'-triphosphate (ATP) by the hexokinase activity in HCCF. The overall mechanism of ocrelizumab reduction is summarized in FIG. 4.

The reducing activity in the HCCF appeared to be transitory in some cases and may be inhibited over time under certain storage conditions or after multiple freeze/thaw cycles. HCCF that has fully lost reducing activity provided an opportunity to explore the role of NADPH and G6P in the reduction of ocrelizumab by Trx system.

An HCCF from a large scale manufacturing run (the "beta" run) was subjected to several freeze/thaw cycles and used in an experiment designed to measure reduction; no ocrelizumab reduction was observed (FIG. 13) despite its ability to bring about antibody reduction seen previously in freshly-thawed HCCF from this same fermentation. NADPH was added to this non-reducing HCCF at a concentration of 5 mM and the reduction event returned (FIG. 14). Therefore, the Trx system is still intact and active in the HCCF where reduction no longer occurs, and capable of reducing protein and/or antibody if supplied with cofactors. Additionally, the reducing activity was lost over time as the NADPH source was depleted (presumably due to the oxidation of NADPH by all of the reductive reactions that compete for NADPH), and not because the Trx system was degraded or inactivated.

This was verified by another experiment. 10 mM G6P was added to a HCCF that had been repeatedly freeze-thawed from the beta run. This G6P addition reactivated the Trx system which subsequently reduced ocrelizumab in the HCCF incubation experiment (FIG. 15). This demonstrated that the reduction of ocrelizumab in the HCCF was caused by the activities of both the Trx system and G6PD. Furthermore,

G6PD is still active in a repeatedly freeze/thawed HCCF of the beta run; the loss of reduction activity in this a repeatedly freeze/thawed HCCF beta run appears to be due to the depletion of G6P, which thus eliminated the conversion of NADP⁺ to NADPH.

In our studies, we have observed that EDTA can effectively inhibit the ocrelizumab reduction in the HCCF incubation experiment. As shown in FIG. 16, the ocrelizumab was reduced after incubating the HCCF from a 12,000 L scale ocrelizumab manufacturing run (not repeatedly freeze/thawed and no loss of reducing activity) at ambient temperature for more than 19 hours. However, the reduction was completely inhibited when 20 mM EDTA was added to the 12 kL HCCF and held in a separate stainless steel minitank (FIG. 17). In the first step of glycolysis, the hexokinase catalyzes the transfer of phosphate group from Mg²⁺-ATP to glucose, a reaction that requires the complexation of Mg²⁺ with ATP (Hammes & Kochavi, 1962a & 1962b, supra). Since EDTA is a metal ion chelator, especially for Mg²⁺, it can be an effective inhibitor of hexokinase. The observation that an excess amount of EDTA can effectively block the reduction indicates the involvement of hexokinase (i.e. providing G6P) in the mechanism of ocrelizumab reduction. Without being bound by this, or any other theory, EDTA blocks the reduction of ocrelizumab by eliminating the hexokinase activity and thereby reducing the G6P level available for G6PD, and subsequently the NADPH level available for the Trx system.

Although EDTA is every effective in blocking the reduction of ocrelizumab in fresh HCCF, it was unable to prevent the reduction of ocrelizumab in the beta run HCCF in which the Trx system activity was lost then reactivated by the addition of G6P. For example, the reduction of ocrelizumab was observed in an HCCF incubation experiment in which 5 mM G6P and 20 mM EDTA (final concentrations) were added to the beta run HCCF that had fully lost reducing activity (FIG. 18). However, no reduction was seen in the control incubation experiment in which no G6P and EDTA were added. Without being bound by this or any other theory, the EDTA used in this manner may therefore inhibit neither the Trx system nor the G6PD, and may function as an inhibitor for hexokinase, which produces the G6P for the G6PD. Without G6P, the Trx system would not be supplied with the necessary NADPH for activity.

Example 6

Inhibition of Reduction of Recombinant Antibody by DHEA

Dehydroepiandrosterone (DHEA), as well as other similar G6PD inhibitors, effectively blocks G6PD activity (Gordon et al., 1995, supra). G6PD inhibitors also prevent the reduction of an antibody in HCCF, for example, ocrelizumab, by blocking the generation of NADPH. The ability of DHEA to inhibit the reduction of ocrelizumab is demonstrated in an HCCF incubation experiment. Adding DHEA to a HCCF prevents antibody reduction.

DHEA is typically used in the concentration range from about 0.05 mM to about 5 mM. DHEA is also typically used in the concentration range from about 0.1 mM to about 2.5 mM.

Example 7

Inhibition of Reduction of Recombinant Antibody by (i) EDTA, (ii) Cupric Sulfate, and (iii) Acetic Acid Additions

Four different HCCFs were stored and held in the stainless steel vials. The solutions were similar in the amount of cell

US 8,574,869 B2

55

lysis, which were generated by diluting HCCF from homogenized CCF with HCCF from non-homogenized CCF. For example, 150 mL of the first lysed solution was mixed with 50 mL of the second solution, respectively. The four HCCF mixtures evaluated in this study contained either: (1) 20 mM EDTA, (2) 30 μ M CuSO₄, (3) 15 mM acetic acid (pH 5.5), and (4) no chemical inhibitor was added for the control solution. The ocrelizumab antibody from all four mixtures was purified immediately (t=0 hr) using protein A chromatography and then again after 20 hr and 40 hr of storage in the stainless steel vials. Purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody (150 kDa). The results showed that greater than 90% intact antibody was present in all four mixtures at the initial time point (FIG. 19). However, at the 20 hr time point, intact antibody was not detected in the control mixture (without any addition) indicating reduction of the antibody disulfide bonds. In the three other mixtures, over 90% intact antibody was still detected at both 20 hr and 40 hr time points, demonstrating the prevention of disulfide bond reduction by all three inhibitors tested.

Example 8

Inhibition of Reduction of Recombinant Antibody by Air Sparging the HCCF

One HCCF mixture generated from homogenized CCF was stored and held in two separate 10 L stainless steel fermentors. One vessel was sparged with air while the other vessel was sparged with nitrogen gas. The ocrelizumab antibody was purified immediately (t=0 hr) from the initial mixture using protein A chromatography. At selected time points,

56

50 mL samples were removed from each vessel and the antibody was purified using protein A chromatography. Purified protein A elution pools were then analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa. The results showed that approximately 85% intact antibody was present in the initial solution (FIG. 20), indicating some early reduction of the antibody disulfide bonds prior to exposure to oxygen (i.e. sparged air in the fermentor). Once the mixture was sparged with air for two hours, greater than 90% intact antibody was measured for the remainder of the 36 hr study. In contrast, when the mixture was sparged with nitrogen gas, the antibody reduction event continued as measured at 2 hr (28% 150 kDa peak) and 6 hr (5% 150 kDa peak). These results demonstrated the prevention of disulfide bond reduction in the antibody when the HCCF mixture generated from homogenized CCF was exposed to oxygen.

Example 9

Design of Targeted siRNA or Antisense Nucleotide Trx Inhibitors

The design of targeted siRNAs or antisense nucleotides to the genes as found in CHO cells may be done by using publicly available sequences such as those for *E. coli* thioredoxin TrxA (SEQ ID NO:30), *E. coli* thioredoxin reductase TrxB (SEQ ID NO:31); mouse thioredoxin 1 (SEQ ID NO:32), mouse thioredoxin 2 (SEQ ID NO:33), mouse thioredoxin reductase 1 (SEQ ID NO:34), and mouse thioredoxin reductase 2 (SEQ ID NO:35). One of ordinary skill in the art can use these sequences to select sequences to design Trx inhibitors for targeting enzymes in different organisms and/or cells, such as CHO cells.

The sequence of *E. coli* Thioredoxin TrxA is:

(SEQ ID NO: 30)

```
ATG TTA CAC CAA CAA CGA AAC CAA CAC GCC AGG CTT ATT CCT GTG GAG
TTA TAT ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC GAC AGT TTT GAC ACG
GAT GTA CTC AAA GCG GAC GGG GCG ATC CTC GTC GAT TTC TGG GCA GAG
TGG TGC GGT CCG TGC AAA ATG ATC GCC CCG ATT CTG GAT GAA ATC GCT
GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAA
AAC CCT GGC ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG
CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG TCT
AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCG TAA.
```

The sequence of *E. coli* Thioredoxin TrxB is:

(SEQ ID NO: 31)

```
ATG GGC ACG ACC AAA CAC AGT AAA CTG CTT ATC CTG GGT TCA GGC CCG
GCG GGA TAC ACC GCT GCT GTC TAC GCG GCG CGC GCC AAC CTG CAA CCT
GTG CTG ATT ACC GGC ATG GAA AAA GGC GGC CAA CTG ACC ACC ACC ACG
GAA GTG GAA AAC TGG CCT GGC GAT CCA AAC GAT CTG ACC GGT CCG TTA
TTA ATG GAG CGC ATG CAC GAA CAT GCC ACC AAG TTT GAA ACT GAG ATC
ATT TTT GAT CAT ATC AAC AAG GTG GAT CTG CAA AAC CGT CCG TTC CGT CTG
AAT GGC GAT AAC GGC GAA TAC ACT TGC GAC GCG CTG ATT ATT GCC ACC
GGA GCT TCT GCA CGC TAT CTC GGC CTG CCC TCT GAA GAA GCC TTT AAA GGC
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US 8,574,869 B2

57

58

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CGT GGG GTT TCT GCT TGT GCA ACC TGC GAC GGT TTC TTC TAT CGC AAC CAG
AAA GTT GCG GTC ATC GGC GGC GGC AAT ACC GCG GTT GAA GAG GCG TTG
TAT CTG TCT AAC ATC GCT TCG GAA GTG CAT CTG ATT CAC CGC CGT GAC GGT
TTC CGC GCG GAA AAA ATC CTC ATT AAG CGC CTG ATG GAT AAA GTG GAG
AAC GGC AAC ATC ATT CTG CAC ACC AAC CGT ACG CTG GAA GAA GTG ACC
GGC GAT CAA ATG GGT GTC ACT GGC GTT CGT CTG CGC GAT ACG CAA AAC
AGC GAT AAC ATC GAG TCA CTC GAC GTT GCC GGT CTG TTT GTT GCT ATC GGT
CAC AGC CCG AAT ACT GCG ATT TTC GAA GGG CAG CTG GAA CTG GAA AAC
GGC TAC ATC AAA GTA CAG TCG GGT ATT CAT GGT AAT GCC ACC CAG ACC
AGC ATT CCT GGC GTC TTT GCC GCA GGC GAC GTG ATG GAT CAC ATT TAT CGC
CAG GCC ATT ACT TCG GCC GGT ACA GGC TGC ATG GCA GCA CTT GAT GCG
GAA CGC TAC CTC GAT GGT TTA GCT GAC GCA AAA TAA.

The sequence of mouse thioredoxin 1 is:

(SEQ ID NO: 32)
ATGGTGAAGCTGATCGAGAGCAAGGAAGCTTTTCAGGAGGCCCTGGCCGC
CGCGGAGACAAGCTTGCTCGTGGTGGACTTCTCTGTCTACGTGGTGTGGAC
CTTGCAAAATGATCAAGCCCTTCTTCCATTCCCTCTGTGACAAGTATTCC
AATGTGGTGTTCCTTGAAGTGGATGTGGATGACTGCCAGGATGTGTCTGC
AGACTGTGAAGTCAAATGCATGCCAGCCTTCCAGTTTTATAAAAAGGGTC
AAAAGTGGGGGAGTTCTCCGGTGCTAACAAGGAAAAGCTTGAAGCCTCT
ATTACTGAATATGCCTAA.

The sequence of mouse thioredoxin 2 is:

(SEQ ID NO: 33)
ATGGCTCAGCGGCTCCTCCTGGGAGGTTCTGACCTCAGTCATCTCCAG
GAAGCCTCCTCAGGGTGTGTGGGCTTCCTCACCTCTAAGACCTGCAGA
CCCCCTCAGTACAATGTGTTGGTCTAACAGTAATGCCAGCCAGCCGCGG
ACAGTACACACCACCAGAGTCTGTTTGACGACCTTTAACGTCCAGGATGG
ACCTGACTTTCAAGACAGAGTTGTCAACAGTGAGACACCAAGTTGTTGTGG
ACTTTTATGCACAGTGGTGTGGCCCTGCAAGATCCTAGGACCGCGGCTA
GAGAAGATGGTCGCCAAGCAGCACGGGAAGGTGGTCATGGCCAAAGTGGA
CATTGACGATCACACAGACCTTGCCATTGAATATGAGGTGTGAGCTGTGC
CTACCGTGCTAGCCATCAAGAACGGGACGTGGTGGACAAGTTTGTGGGG
ATCAAGGACGAGGACCAGCTAGAAGCCTTCTGAAGAAGCTGATTGGCTG
A.

The sequence of mouse thioredoxin reductase 1 is:

(SEQ ID NO: 34)
ATGAATGGCTCCAAAGATCCCCCTGGGTCTATGACTTCGACCTGATCAT
CATTGGAGGAGGCTCAGGAGGACTGGCAGCAGCTAAGGAGGCAGCCAAAT
TTGACAAGAAAGTGTGGTCTTGATTCTGTACACCGACTCCTCTTGGG

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ACCAGATGGGGTCTCGGAGGAACGTGTGTGAATGTGGGTGCATACCTAA
GAAGCTGATGCACCAGGCAGCTTTGCTCGGACAAGCTCTGAAAGACTCGC
GCAACTATGGCTGGAAAGTCGAAGACACAGTGAAGCATGACTGGGAGAAA
ATGACGGAATCTGTGCAGAGTCACATCGGCTCGCTGAAGTGGGGTACCG
CGTAGCTCTCCGGGAGAAAAAGTCTGTATGAGAATGCTTACGGGAGGT
TCATTGGTCTTCCAGGATTTGTGGCGACAAATAACAAAGGTAAGAAAAA
ATCTATTAGCAGAGCGGTTCTCTCATCGCCACAGGTGAGAGGCCCGCTA
CCTGGGCATCCCTGGAGACAAAGAGTACTGCATCAGCAGTGATGATCTTT
TCTCCTTGCCCTTACTGCCCGGGAAGACCTAGTAGTTGGTGCATCCTAT
GTCGCCCTTGAATGTGAGGATTTCTGGCTGGTATCGGCTTAGAGCTCAC
TGTAATGGTGCAGTCCATTCTCCTTAGAGGATTTGACCAAGACATGGCCA
ACAAAATCGGTGAACACATGGAAGAATCGGTATCAAGTTTATAAGGCAG
TTCGTCCCAACGAAAATGAACAGATCGAAGCAGGAACACAGGCGGACT
CAGGGTGACTGCTCAATCCACAAACAGCGAGGAGACCATAGAGGGCGAAT
TTAACACAGTGTGTGCTGGCGGTAGGAAGAGATTCTTGTACGAGAACTATT
GGCTTAGAGACCGTGGGCGTGAAGATAAACGAAAAACCGGAAGATACC
CGTCACGGATGAAGAGCAGACCAATGTGCCTTACATCTACGCCATCGGTG
ACATCCTGGAGGGGAAGCTAGAGCTGACTCCCGTAGCCATCCAGGCGGGG
AGATTGCTGGCTCAGAGGCTGTATGGAGGCTCCAATGTCAAATGTGACTA
TGACAATGTCCCAACGACTGTATTTACTCCTTTGGAATATGGCTGTTGTG
GCCTCTCTGAAGAAAAGCCGTAGAGAAATTTGGGAAGAAAATATTGAA
GTTTACCATAGTTTCTTTTGGCCATTGGAATGGACAGTCCCATCCCGGGA
TAACAACAAATGTATGCAAAAATAATCTGCAACCTTAAAGACGATGAAC
GTGTCGTGGGCTTCCACGTGCTGGGTCCAAACGCTGGAGAGGTGACGCAG
GGCTTTGCGGCTGCGCTCAAGTGTGGGCTGACTAAGCAGCAGCTGGACAG
CACCATCGGCATCCACCCGGTCTGTGCAGAGATATTACAACGTTGTGAG
TGACGAAGCGCTCTGGGGGAGACATCCTCCAGTCTGGCTGCTGA

US 8,574,869 B2

59

The sequence of mouse thioredoxin reductase 2 is:

(SEQ ID NO: 35)

ATGGCGGCGATGGTGGCGGCGATGGTGGCGGCGCTGCGTGGACCCAGCAG
 GCGCTTCCGGCCCGGACACGGGCTCTGACACGCGGGACAAGGGCGCGG
 CGAGTGACGCGGGAGGGCAGCAGAGCTTTGATCTCTTGGTGATCGGTGGG
 GGATCCGGTGGCCTAGCTTGTGCCAAGGAAGCTGCTCAGCTGGGAAAGAA
 GGTGGCTGTGGTGACTATGTGGAACCTTCTCCCGAGGCACCAAGTGGG
 GCCTTGGTGGCACCTGTGTCAACGTGGGTTGCATACCCAAGAAGCTGATG
 CATCAGGCTGCACCTGCTGGGGGCGATGATCAGAGATGCTCACCACATATGG
 CTGGGAGGTGGCCAGCCTGTCCAACACAACCTGGAAGACAATGCGAGAAG
 CCGTGCAAAACCATGTGAAATCCTTGAACCTGGGGTCATCGCGTCCAACCTG
 CAGGACAGGAAAGTCAAGTACTTTAACATCAAAGCCAGCTTTGTGGATGA
 GCACACAGTTTCGCGGTGTGGACAAAGCGGGAAGGCGACTCTGCTTTTTCAG
 CTGAGCACATTGTCTTGTCTACAGGAGGACGGCCAAGGTACCCACACAA
 GTCAAAGGAGCCCTGGAATATGGAATCACAAGTGACGACATCTTCTGGCT
 GAAGGAGTCCCTGGGAAAACGTTGGTGGTGGAGCCAGCTATGTGGCCC
 TAGAGTGTGCTGGCTTCTCACTGGAATTGGACTGGATACCACTGTCTATG
 ATGCGCAGCATCCCTCTCCGAGGCTTTGACAGCAAATGTCTATCTTTGGT
 CACAGAGCACATGGAGTCTCATGGCACCCAGTTCTCTGAAAGGCTGTGTCC
 CCTCCACATCAAAAACTCCCACTAACCAGCTGCAGGTCACTTGGGAG
 GATCATGCTTCTGGCAAGGAAGACACAGGCACCTTTGACACTGTCTGTG
 GGCCATAGGGCGAGTTCCAGAAACCAGGACTTTGAATCTGGAGAAGGCTG
 GCATCAGTACCAACCTAAGAATCAGAAGATTATTGTGGATGCCAGGAG
 GCTACCTCTGTTCCTCCACATCTATGCCATTGGAGATGTTGTGAGGGGCG
 GCCTGAGCTGACGCCACAGCTATCAAGGCAGGAAAGCTTCTGGCTCAGC
 GGCTCTTTGGGAAATCCTCAACCTTAATGGATTACAGCAATGTTCCACA
 ACTGTCTTTACACCACTGGAGTATGGCTGTGTGGGGCTGTCTGAGGAGGA
 GGCTGTGGCTCTCCATGGCCAGGAGCATGTAGAGGTTTACCATGCATATT
 ATAAGCCCTAGAGTTACGGTGGCGGATAGGGATGCATCAGTGCTAC
 ATAAAGATGGTATGCATGAGGGAGCCCCACAACCTGGTGTGGGCTGCA
 CTTCTTGGCCCCAACGCTGGAGAAGTCACCAAGGATTTGCTCTTGGGA
 TCAAGTGTGGGGCTTCATATGCACAGGTGATGCAGACAGTAGGGATCCAT
 CCCACCTGCTCTGAGGAGGTGGTCAAGCTGCACATCTCCAAGCGCTCCGG
 CCTGGAGCCTACTGTGACTGGTTGCTGA.

Example 10

In Vitro Trx/Trx Reductase Studies

Materials and Methods

A commercial TrxR (rat liver) solution (4 μ M) was diluted with water to yield a 2.86 μ M solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μ M solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

60

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μ L reaction buffer (10 mM histidine, 10 mM Na₂SO₄, 137 mM NaCl, 2.5 mM KCl, pH 7.0), 25 μ L NADPH, 16 μ L formulated ocrelizumab solution (30.2 mg/mL) and 5 μ L Trx were gently mixed. The reaction was initiated by the addition of 17.5 μ L TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μ L were taken at each sampling time-point and stored at -70° C. until analyzed by the Bioanalyzer assay.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of various inhibitors.

1. In Vitro Activity of Thioredoxin System

FIG. 24 shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, referred to as "Variant A" above) (1 mg/mL) with 0.1 μ M TrxR (rat liver), 5 μ M Trx (human) and 1 mM NADPH in 10 mM histidine sulfate buffer results in the reduction of ocrelizumab in less than one hour.

2. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose

Aurothioglucose (ATG) was added to the ocrelizumab mixture described above, at the following concentrations: 1 mM; 0.6 μ M (6:1 ATG:TrxR); 0.4 μ M (4:1 ATG:TrxR); and 0.2 μ M (2:1 ATG:TrxR).

As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. 25-27, aurothioglucose added at concentrations 1 mM, 0.6 μ M, and 0.4 μ M effectively inhibits the reduction of ocrelizumab by the thioredoxin system. However, as shown in FIG. 28, under these experimental conditions aurothioglucose added at a concentration of 0.2 μ M cannot inhibit ocrelizumab reduction after 24 hours.

3. In Vitro Activity of Thioredoxin System Inhibited by Aurothiomalate

Aurothiomalate (ATM) was added to the ocrelizumab mixture described above, at concentrations of 0.1 mM and 0.01 mM. As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. 29 and 30, ATM effectively inhibits the reduction of ocrelizumab by the thioredoxin system at both concentrations tested.

4. In Vitro Activity of Thioredoxin System Inhibited by CuSO₄

CuSO₄ was added to the ocrelizumab mixture described above, at concentrations of 20 μ M (4:1 Cu²⁺:Trx); 10 μ M (2:1 Cu²⁺:Trx); and 5 μ M (1:1 Cu²⁺:Trx). As shown in FIGS. 31-33, CuSO₄ effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 20 μ M and 10 μ M (FIGS. 31 and 32), but the 5 μ M concentration is insufficient to result in a complete inhibition of reduction (FIG. 33).

5. In Vitro Activity of Thioredoxin System Inhibited by Cystamine

Cystamine was added to the ocrelizumab mixture described above at the following concentrations: 532 μ M (20:1 cystamine:2H7 (Variant A) disulfide); 266 μ M (10:1 cystamine:2H7 (Variant A) disulfide); 133 μ M (5:1 cystamine:2H7 disulfide); and 26.6 μ M (1:1 cystamine:2H7 (Variant A) disulfide). As shown in FIGS. 34-37, cystamine effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 532 μ M (20:1 cystamine:2H7 (Variant A) disulfide) and 266 μ M (10:1 cystamine:2H7 (Variant A) disulfide) (FIGS. 34 and 35) but the 133 μ M (5:1 cystamine:2H7 (Variant A) disulfide) and 26.6 μ M (1:1 cystamine:2H7 (Variant A) disulfide) concentrations are insufficient to inhibit the reduction of ocrelizumab after 24 hours (FIGS. 36 and 37).

6. In Vitro Activity of Thioredoxin System Inhibited by Cystine

Cystine was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. 38, at this concentration cystine effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted that the minimum effective concentration of cystine (just as the effective minimum concentration of other inhibitors) depends on the actual circumstances, and might be different for different proteins, such as antibodies, and might vary depending on the timing of addition. Thus, for example, if cystine is added pre-lysis, the minimum effective concentration for antibody 2H7 (Variant A) is about 1.3 mM, for Apomab about 1 mM and for antibody Variant C about 4.5 mM. When cystine is added in the cell culture medium, the minimum effective concentration typically is somewhat higher, and is about 5.2 mM for 2H7 (Variant A), 6 mM for Apomab and 9 mM for antibody Variant C. Usually, for cystine, cystamine and oxidized glutathione (see below) the minimum effective inhibitory concentration is about 40x of the antibody concentration (in μ M).

7. In Vitro Activity of Thioredoxin System Inhibited by Oxidized Glutathione (GSSG)

GSSG was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. 39, at this concentration GSSG effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted, however, that the minimum effective concentration of oxidized glutathione (just as that of the other inhibitors) depends on the actual circumstances, such as, for example, on the nature of the protein (e.g. antibody) produced and the timing of addition. For example, for antibody 2H7 (Variant A) the minimum effective concentration is about 1.3 mM for addition prior to lysis.

8. In Vitro Activity of Enzymatic Reduction System

FIG. 40 shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, Variant A) (1 mg/mL) with 10 μ g/mL hexokinase, 50 μ g/mL glucose-6-phosphate dehydrogenase, 5 μ M thioredoxin, 0.1 μ M thioredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+} , and 2 mM NADP in 50 mM histidine sulfate buffered at pH 7.38 results in the reduction of ocrelizumab in about one hour. Addition of 0.1 mM HDEA, a known glucose-6-phosphate dehydrogenase inhibitor does not inhibit the reduction.

9. In Vitro Activity of Enzymatic Reduction System Requires NADPH

As shown in the digital gel-like image from Bioanalyzer analysis of FIG. 41, incubation of intact ocrelizumab (1 mg/mL) with 5 μ M thioredoxin, 0.1 μ M thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH 7.38 does not result in the reduction of the ocrelizumab antibody. Reduction of ocrelizumab could not occur without hexokinase and glucose-6-phosphate dehydrogenase and their substrates to generate NADPH.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein.

Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form the part of these inventions. This includes within the generic description of each of the inventions a proviso or negative limitation that will allow removing any subject matter from the genus, regardless of whether or not the material to be removed was specifically recited. In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further, when a reference to an aspect of the invention lists a range of individual members, as for example, 'SEQ ID NO:1 to SEQ ID NO:100, inclusive,' it is intended to be equivalent to listing every member of the list individually, and additionally it should be understood that every individual member may be excluded or included in the claim individually.

The steps depicted and/or used in methods herein may be performed in a different order than as depicted and/or stated. The steps are merely exemplary of the order these steps may occur. The steps may occur in any order that is desired such that it still performs the goals of the claimed invention.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 35

<210> SEQ ID NO 1

<211> LENGTH: 107

<212> TYPE: PRT

US 8,574,869 B2

63

64

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain variable

<400> SEQUENCE: 1

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1           5           10           15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
           20           25           30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
           35           40           45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
           50           55           60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65           70           75           80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
           85           90           95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
           100           105

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<210> SEQ ID NO 2
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 2

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1           5           10           15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
           20           25           30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
           35           40           45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
           50           55           60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65           70           75           80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
           85           90           95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
           100           105           110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
           115           120

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<210> SEQ ID NO 3
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain variable

<400> SEQUENCE: 3

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1           5           10           15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
           20           25           30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
           35           40           45

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US 8,574,869 B2

65

66

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Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

<210> SEQ ID NO 4
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 4

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 5
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 5

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

US 8,574,869 B2

67

68

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<210> SEQ ID NO 6
<211> LENGTH: 213
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

<400> SEQUENCE: 6
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1           5           10           15
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
20           25           30
His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35           40           45
Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50           55           60
Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65           70           75           80
Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
85           90           95
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100          105          110
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115          120          125
Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130          135          140
Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145          150          155          160
Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165          170          175
Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180          185          190
Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195          200          205
Asn Arg Gly Glu Cys
210

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<210> SEQ ID NO 7
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 7
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1           5           10           15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20           25           30
Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35           40           45
Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
50           55           60
Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65           70           75           80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85           90           95

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US 8,574,869 B2

69

70

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Ala	Arg	Val	Val	Tyr	Tyr	Ser	Asn	Ser	Tyr	Trp	Tyr	Phe	Asp	Val	Trp
			100					105					110		
Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro
		115					120					125			
Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr
	130					135					140				
Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr
145					150					155					160
Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro
			165					170						175	
Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr
		180						185					190		
Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn
	195						200					205			
His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser
	210					215					220				
Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu
225					230					235					240
Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu
			245					250						255	
Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser
		260						265					270		
His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu
	275						280					285			
Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr
	290					295					300				
Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn
305					310					315					320
Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro
			325					330						335	
Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln
	340						345						350		
Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val
	355						360					365			
Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val
	370					375				380					
Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro
385					390					395					400
Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr
			405					410						415	
Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val
		420						425					430		
Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu
	435						440					445			
Ser	Pro	Gly	Lys												
	450														

<210> SEQ ID NO 8
 <211> LENGTH: 452
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: full length heavy chain

 <400> SEQUENCE: 8

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly

US 8,574,869 B2

71

72

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1	5	10	15
Ser Leu Arg	Leu Ser Cys Ala Ala	Ser Gly Tyr Thr Phe Thr	Ser Tyr
	20	25	30
Asn Met His	Trp Val Arg Gln Ala Pro	Gly Lys Gly Leu Glu Trp Val	
	35	40	45
Gly Ala Ile	Tyr Pro Gly Asn Gly Asp Thr Ser	Tyr Asn Gln Lys Phe	
	50	55	60
Lys Gly Arg	Phe Thr Ile Ser Val Asp Lys	Ser Lys Asn Thr Leu Tyr	
	65	70	75
Leu Gln Met	Asn Ser Leu Arg Ala Glu Asp Thr	Ala Val Tyr Tyr Cys	
	85	90	95
Ala Arg Val	Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr	Phe Asp Val Trp	
	100	105	110
Gly Gln Gly	Thr Leu Val Thr Val Ser Ser Ala Ser	Thr Lys Gly Pro	
	115	120	125
Ser Val Phe	Pro Leu Ala Pro Ser Ser Lys Ser	Thr Ser Gly Gly Thr	
	130	135	140
Ala Ala Leu	Gly Cys Leu Val Lys Asp Tyr Phe	Pro Glu Pro Val Thr	
	145	150	155
Val Ser Trp	Asn Ser Gly Ala Leu Thr Ser Gly	Val His Thr Phe Pro	
	165	170	175
Ala Val Leu	Gln Ser Ser Gly Leu Tyr Ser Leu Ser	Ser Val Val Thr	
	180	185	190
Val Pro Ser	Ser Ser Leu Gly Thr Gln Thr Tyr Ile	Cys Asn Val Asn	
	195	200	205
His Lys Pro	Ser Asn Thr Lys Val Asp Lys Lys	Val Glu Pro Lys Ser	
	210	215	220
Cys Asp Lys	Thr His Thr Cys Pro Pro Cys Pro	Ala Pro Glu Leu Leu	
	225	230	235
Gly Gly Pro	Ser Val Phe Leu Phe Pro Pro Lys	Pro Lys Asp Thr Leu	
	245	250	255
Met Ile Ser	Arg Thr Pro Glu Val Thr Cys Val Val	Val Asp Val Ser	
	260	265	270
His Glu Asp	Pro Glu Val Lys Phe Asn Trp Tyr Val	Asp Gly Val Glu	
	275	280	285
Val His Asn	Ala Lys Thr Lys Pro Arg Glu Glu	Gln Tyr Asn Ala Thr	
	290	295	300
Tyr Arg Val	Val Ser Val Leu Thr Val Leu His	Gln Asp Trp Leu Asn	
	305	310	315
Gly Lys Glu	Tyr Lys Cys Lys Val Ser Asn Lys	Ala Leu Pro Ala Pro	
	325	330	335
Ile Ala Ala	Thr Ile Ser Lys Ala Lys Gly Gln Pro	Arg Glu Pro Gln	
	340	345	350
Val Tyr Thr	Leu Pro Pro Ser Arg Glu Glu Met	Thr Lys Asn Gln Val	
	355	360	365
Ser Leu Thr	Cys Leu Val Lys Gly Phe Tyr Pro	Ser Asp Ile Ala Val	
	370	375	380
Glu Trp Glu	Ser Asn Gly Gln Pro Glu Asn Asn	Tyr Lys Thr Thr Pro	
	385	390	395
Pro Val Leu	Asp Ser Asp Gly Ser Phe Phe Leu	Tyr Ser Lys Leu Thr	
	405	410	415
Val Asp Lys	Ser Arg Trp Gln Gln Gly Asn Val	Phe Ser Cys Ser Val	
	420	425	430

US 8,574,869 B2

73

74

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Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
435 440 445

Ser Pro Gly Lys
450

<210> SEQ ID NO 9
<211> LENGTH: 213
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

<400> SEQUENCE: 9

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
210

<210> SEQ ID NO 10
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 10

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
50 55 60

US 8,574,869 B2

75

76

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Lys	Gly	Arg	Phe	Thr	Ile	Ser	Val	Asp	Lys	Ser	Lys	Asn	Thr	Leu	Tyr	65	70	75	80
Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	85	90	95	
Ala	Arg	Val	Val	Tyr	Tyr	Ser	Ala	Ser	Tyr	Trp	Tyr	Phe	Asp	Val	Trp	100	105	110	
Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	115	120	125	
Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	130	135	140	
Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	145	150	155	160
Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	165	170	175	
Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	180	185	190	
Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	195	200	205	
His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	210	215	220	
Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	225	230	235	240
Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	245	250	255	
Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	260	265	270	
His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	275	280	285	
Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ala	Thr	290	295	300	
Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	305	310	315	320
Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	325	330	335	
Ile	Ala	Ala	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	340	345	350	
Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val	355	360	365	
Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	370	375	380	
Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	385	390	395	400
Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	405	410	415	
Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	420	425	430	
Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	435	440	445	
Ser	Pro	Gly	Lys													450			

<210> SEQ ID NO 11

<211> LENGTH: 452

<212> TYPE: PRT

<213> ORGANISM: Artificial

US 8,574,869 B2

77

78

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<220> FEATURE:

<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 11

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
 20 25 30
 Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
 100 105 110
 Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
 115 120 125
 Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
 130 135 140
 Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
 145 150 155 160
 Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
 165 170 175
 Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
 180 185 190
 Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
 195 200 205
 His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
 210 215 220
 Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
 225 230 235 240
 Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
 245 250 255
 Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
 260 265 270
 His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
 275 280 285
 Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
 290 295 300
 Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
 305 310 315 320
 Gly Lys Glu Tyr Lys Cys Ala Val Ser Asn Lys Ala Leu Pro Ala Pro
 325 330 335
 Ile Glu Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
 340 345 350
 Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
 355 360 365
 Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
 370 375 380
 Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
 385 390 395 400

80

Glu 1	Val	Gln	Leu 5	Val	Glu	Ser	Gly	Gly 10	Leu	Val	Gln	Pro	Gly 15	Gly
Ser	Leu	Arg	Leu 20	Ser	Cys	Ala	Ala	Ser 25	Gly	Tyr	Thr	Phe	Thr 30	Tyr
Asn	Met	His 35	Trp	Val	Arg	Gln	Ala 40	Pro	Gly	Lys	Gly	Leu 45	Glu	Trp
Gly	Ala 50	Ile	Tyr	Pro	Gly	Asn 55	Gly	Ala	Thr	Ser	Tyr 60	Asn	Gln	Lys
Lys 65	Gly	Arg	Phe	Thr	Ile 70	Ser	Val	Asp	Lys	Ser 75	Lys	Asn	Thr	Leu
Leu	Gln	Met	Asn 85	Ser	Leu	Arg	Ala	Glu	Asp 90	Thr	Ala	Val	Tyr	Tyr
Ala	Arg	Val	Val 100	Tyr	Tyr	Ser	Ala	Ser 105	Tyr	Trp	Tyr	Phe	Asp 110	Val
Gly	Gln	Gly 115	Thr	Leu	Val	Thr	Val 120	Ser	Ser	Ala	Ser	Thr 125	Lys	Gly
Ser	Val 130	Phe	Pro	Leu	Ala	Pro 135	Ser	Ser	Lys	Ser	Thr 140	Ser	Gly	Gly
Ala 145	Ala	Leu	Gly	Cys	Val 150	Lys	Asp	Tyr	Phe 155	Pro	Glu	Pro	Val	Thr
Val	Ser	Trp	Asn 165	Ser	Gly	Ala	Leu	Thr	Ser 170	Gly	Val	His	Thr 175	Pro
Ala	Val	Leu 180	Gln	Ser	Ser	Gly	Leu 185	Tyr	Ser	Leu	Ser	Ser	Val 190	Thr
Val	Pro 195	Ser	Ser	Ser	Leu	Gly	Thr 200	Gln	Thr	Tyr	Ile	Cys 205	Asn	Val
His 210	Lys	Pro	Ser	Asn	Thr 215	Lys	Val	Asp	Lys	Lys 220	Val	Glu	Pro	Lys
Cys 225	Asp	Lys	Thr	His	Thr 230	Cys	Pro	Pro	Cys	Pro 235	Ala	Pro	Glu	Leu
Gly	Gly	Pro	Ser 245	Val	Phe	Leu	Phe	Pro 250	Pro	Lys	Pro	Lys	Asp 255	Leu
Met	Ile	Ser	Arg 260	Thr	Pro	Glu	Val	Thr 265	Cys	Val	Val	Val	Asp 270	Ser
His	Glu 275	Asp	Pro	Glu	Val	Lys	Phe 280	Asn	Trp	Tyr	Val	Asp 285	Gly	Val
Val 290	His	Asn	Ala	Lys	Thr	Lys 295	Pro	Arg	Glu	Glu	Gln 300	Tyr	Asn	Ala
Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu

US 8,574,869 B2

81

82

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305          310          315          320
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
          325          330          335
Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
          340          345          350
Val Tyr Thr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
          355          360          365
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
          370          375          380
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385          390          395          400
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
          405          410          415
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
          420          425          430
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
          435          440          445
Ser Pro Gly Lys
          450

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<210> SEQ ID NO 13
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

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<400> SEQUENCE: 13

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1          5          10          15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
          20          25          30
Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
          35          40          45
Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
          50          55          60
Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65          70          75          80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
          85          90          95
Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
          100          105          110
Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
          115          120          125
Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
          130          135          140
Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145          150          155          160
Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
          165          170          175
Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
          180          185          190
Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
          195          200          205
His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
210          215          220

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US 8,574,869 B2

83

84

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Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
 225 230 235 240
 Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
 245 250 255
 Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
 260 265 270
 His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
 275 280 285
 Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
 290 295 300
 Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
 305 310 315 320
 Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
 325 330 335
 Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
 340 345 350
 Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
 355 360 365
 Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
 370 375 380
 Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
 385 390 395 400
 Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
 405 410 415
 Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
 420 425 430
 Met His Glu Ala Leu His Trp His Tyr Thr Gln Lys Ser Leu Ser Leu
 435 440 445
 Ser Pro Gly Lys
 450

<210> SEQ ID NO 14
 <211> LENGTH: 452
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 14

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
 20 25 30
 Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
 100 105 110
 Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
 115 120 125

US 8,574,869 B2

85

86

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Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
 130 135 140
 Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
 145 150 155 160
 Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
 165 170 175
 Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
 180 185 190
 Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
 195 200 205
 His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
 210 215 220
 Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
 225 230 235 240
 Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
 245 250 255
 Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
 260 265 270
 His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
 275 280 285
 Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
 290 295 300
 Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
 305 310 315 320
 Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
 325 330 335
 Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
 340 345 350
 Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
 355 360 365
 Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
 370 375 380
 Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
 385 390 395 400
 Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
 405 410 415
 Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
 420 425 430
 Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
 435 440 445
 Ser Pro Gly Lys
 450

<210> SEQ ID NO 15
 <211> LENGTH: 452
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: full length heavy chain

 <400> SEQUENCE: 15

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
 20 25 30
 Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val

88

	35					40					45				
Gly	Ala	Ile	Tyr	Pro	Gly	Asn	Gly	Asp	Thr	Ser	Tyr	Asn	Gln	Lys	Phe
	50					55					60				
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Val	Asp	Lys	Ser	Lys	Asn	Thr	Leu	Tyr
65					70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
				85					90					95	
Ala	Arg	Val	Val	Tyr	Tyr	Ser	Asn	Ser	Tyr	Trp	Tyr	Phe	Asp	Val	Trp
				100					105					110	
Gly	Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro
				115				120					125		
Ser	Val	Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr
	130					135					140				
Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr
145					150					155					160
Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro
				165					170					175	
Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr
			180					185					190		
Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn
	195						200					205			
His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser
	210					215					220				
Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu
225					230					235					240
Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu
				245					250					255	
Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser
			260					265					270		
His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu
	275						280					285			
Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ala	Thr
	290					295					300				
Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn
305					310					315					320
Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Ala	Ala	Leu	Pro	Ala	Pro
				325					330					335	
Ile	Ala	Ala	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln
			340					345					350		
Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val
	355						360					365			
Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val
	370					375					380				
Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro
385					390					395					400
Pro</															

US 8,574,869 B2

89

90

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<210> SEQ ID NO 16
 <211> LENGTH: 107
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: antibody

<400> SEQUENCE: 16

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ile Gly
 20 25 30
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45
 Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr
 85 90 95
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
 100 105

<210> SEQ ID NO 17
 <211> LENGTH: 119
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: antibody

<400> SEQUENCE: 17

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr Asp Tyr
 20 25 30
 Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
 50 55 60
 Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr Trp Gly Gln Gly
 100 105 110
 Thr Leu Val Thr Val Ser Ser
 115

<210> SEQ ID NO 18
 <211> LENGTH: 107
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: light chain

<400> SEQUENCE: 18

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala
 20 25 30

US 8,574,869 B2

91

92

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Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60

Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro
 85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
 100 105

<210> SEQ ID NO 19
 <211> LENGTH: 120
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 19

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr
 20 25 30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr
 65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95

Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln
 100 105 110

Gly Thr Leu Val Thr Val Ser Ser
 115 120

<210> SEQ ID NO 20
 <211> LENGTH: 108
 <212> TYPE: PRT
 <213> ORGANISM: artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: antibody

<400> SEQUENCE: 20

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
 1 5 10 15

Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
 20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Val Leu Ile
 35 40 45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60

Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro
 65 70 75 80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
 85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
 100 105

US 8,574,869 B2

93

94

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<210> SEQ ID NO 21
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 21
Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly Glu
1             5             10             15
Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
                20             25             30
Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met
35             40             45
Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
50             55             60
Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65             70             75             80
Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp Thr Ala Thr Tyr Phe Cys
85             90             95
Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
100            105            110
Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser
115            120

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<210> SEQ ID NO 22
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 22
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1             5             10             15
Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
20            25            30
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
35            40            45
Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50            55            60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65            70            75            80
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
85            90            95
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100           105

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<210> SEQ ID NO 23
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 23
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1             5             10             15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr

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US 8,574,869 B2

95

96

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	20		25		30
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val					
35			40		45
Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe					
50			55		60
Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr					
65			70		75
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys					
			85		90
Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val					
			100		105
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser					
115			120		

<210> SEQ ID NO 24
 <211> LENGTH: 108
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: antibody

<400> SEQUENCE: 24

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly					
1			5		10
Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr					
			20		25
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile					
			35		40
Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly					
			50		55
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro					
65			70		75
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp					
			85		90
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg					
			100		105

<210> SEQ ID NO 25
 <211> LENGTH: 123
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: antibody

<400> SEQUENCE: 25

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly					
1			5		10
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr His Tyr					
			20		25
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val					
			35		40
Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe					
			50		55
Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr					
65			70		75
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys					
			85		90
Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val					

US 8,574,869 B2

97

98

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100	105	110
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Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 26
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 26

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

<210> SEQ ID NO 27
<211> LENGTH: 121
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 27

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
20 25 30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
50 55 60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 28
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 28

US 8,574,869 B2

99

100

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
 20 25 30
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45
 Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
 85 90 95
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
 100 105 110
 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
 115 120 125
 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
 130 135 140
 Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
 145 150 155 160
 Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
 165 170 175
 Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
 180 185 190
 Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
 195 200 205
 Phe Asn Arg Gly Glu Cys
 210

<210> SEQ ID NO 29
 <211> LENGTH: 451
 <212> TYPE: PRT
 <213> ORGANISM: Artificial
 <220> FEATURE:
 <223> OTHER INFORMATION: antibody

<400> SEQUENCE: 29

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
 20 25 30
 Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
 50 55 60
 Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
 100 105 110
 Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
 115 120 125
 Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala
 130 135 140
 Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val

US 8,574,869 B2

101

102

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145	150	155	160
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala	165	170	175
Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val	180	185	190
Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His	195	200	205
Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys	210	215	220
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly	225	230	235
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met	245	250	255
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His	260	265	270
Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val	275	280	285
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr	290	295	300
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly	305	310	315
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile	325	330	335
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val	340	345	350
Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser	355	360	365
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu	370	375	380
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro	385	390	395
Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val	405	410	415
Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met	420	425	430
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser	435	440	445
Pro Gly Lys	450		

<210> SEQ ID NO 30

<211> LENGTH: 384

<212> TYPE: DNA

<213> ORGANISM: E. coli Thioredoxin TrxA

<400> SEQUENCE: 30

atgttacacc aacaacgaaa ccaacacgcc aggccttattc ctgtggagtt atatatgagc	60
gataaaatta ttacactgac tgacgacagt ttgacacgg atgtactcaa agcggacggg	120
gcgatcctcg tcgatttctg ggcagagtgg tgcgggccgt gcaaaatgat cgcctcgatt	180
ctggatgaaa tcgctgacga atatcagggc aaactgaccg ttgcaaaact gaacatcgat	240
caaaaccctg gcactgcgcc gaaatatggc atccgtggta tcccgactct gctgctgttc	300
aaaaacggtg aagtggcggc aaccaaagtg ggtgcactgt ctaaaggtea gttgaaagag	360
ttctctgacg ctaacctggc gtaa	384

US 8,574,869 B2

103

104

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<210> SEQ ID NO 31
 <211> LENGTH: 966
 <212> TYPE: DNA
 <213> ORGANISM: E. coli thioredoxin reductase TrxB

<400> SEQUENCE: 31

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atgggcacga ccaaacacag taaactgctt atcctggggt caggcccggc gggatacacc    60
gctgctgtct acgcggcgcg cgccaacctg caacctgtgc tgattaccgg catggaaaaa    120
ggcgcccaac tgaccaccac cacggaagtg gaaaactggc ctggcgatcc aaacgatctg    180
accggtcctg tattaatgga gcgcatgcac gaacatgcc acaagtttga aactgagatc    240
atttttgatc atatcaacaa ggtggatctg caaaaccgtc cgttcctgtc gaatggcgat    300
aacggcgaat acacttgcca gcgctgatt attgccaccg gagcttctgc acgtatctc    360
ggcctgcctc ctgaagaagc ctttaaaggc cgtgggggtt ctgcttgtgc aacctgcgac    420
ggttttctct atcgcaacca gaaagtgtcg gtcacggcg gcggaatac cgcggttgaa    480
gaggcggtgt atctgtctaa catcgcttcg gaagtgcac tgattaccg ccgtgacggg    540
ttcgcgcgcg aaaaaatcct cattaagcgc ctgatggata aagtggagaa cggcaacatc    600
attctgcaca ccaaccgtac gctggaagaa gtgaccggcg atcaaatggg tgtcactggc    660
gttcgtctgc gcgatacgca aaacagcgat aacatcgagt cactcgacgt tgcgggtctg    720
ttgttgctta tcggtcacag cccgaatact gcgattttcg aagggcagct ggaactggaa    780
aacggctaca tcaaagtaca gtcgggtatt catggtaatg ccaaccagac cagcattcct    840
ggcgctcttg ccgcaggcga cgtgatggat cacatttatc gccaggccat tacttcggcc    900
ggtacaggct gcatggcagc acttgatgcg gaacgctacc tcgatgggtt agctgacgca    960
aaataa                                           966
  
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<210> SEQ ID NO 32
 <211> LENGTH: 318
 <212> TYPE: DNA
 <213> ORGANISM: mus musculus

<400> SEQUENCE: 32

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atggtgaagc tgatcgagag caaggaagct tttcaggagg ccctggccgc cgcgggagac    60
aagcttgctg tgggtgactt ctctgctacg tggtgtggac cttgcaaaat gatcaagccc    120
ttcttccatt ccctctgtga caagtattcc aatgtggtgt tccttgaagt ggatgtggat    180
gactgccagg atgttgctgc agactgtgaa gtcaaatgca tgccgacctt ccagttttat    240
aaaaagggtc aaaagggtgg ggagttctcc ggtgctaaca aggaaaagct tgaagcctct    300
attactgaat atgcctaa                                           318
  
```

<210> SEQ ID NO 33
 <211> LENGTH: 501
 <212> TYPE: DNA
 <213> ORGANISM: mus musculus

<400> SEQUENCE: 33

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atggctcagc ggctcctcct ggggaggttc ctgacctcag tcactctccag gaagcctcct    60
cagggtgtgt gggcttcctc cacctetaag accctgcaga cccctcagta caatgctggt    120
ggtctaacag taatgcccg cccagcccg acagtacaca ccaccagagt ctgtttgacg    180
acctttaacg tccaggatgg acctgacttt caagacagag ttgtcaacag tgagacacca    240
gttggttggt actttcatgc acagtgggtg gggccctgca agatcctagg accgcggtta    300
  
```

US 8,574,869 B2

105

106

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gagaagatgg tcgccaagca gcacgggaag gtggatcatgg ccaaagtga cattgacgat	360
cacacagacc ttgccattga atatgaggtg tcagctgtgc ctaccgtgct agccatcaag	420
aacggggacg tggtaggaaa gttttagggg atcaaggacg aggaccagct agaagccttc	480
ctgaagaagc tgattggctg a	501

<210> SEQ ID NO 34
 <211> LENGTH: 1494
 <212> TYPE: DNA
 <213> ORGANISM: mus musculus

<400> SEQUENCE: 34

atgaatggct ccaaagatcc ccttgggtcc tatgacttcg acctgatcat cattggagga	60
ggctcaggag gactggcagc agctaaggag gcagccaaat ttgacaagaa agtgcctggtc	120
ttggattttg tcacaccgac tcctcttggg accagatggg gtctcggagg aacgtgtgtg	180
aatgtgggtt gcatacctaa gaagctgatg caccaggcag ctttgcctcg acaagctctg	240
aaagactcgc gcaactatgg ctggaaagtc gaagacacag tgaagcatga ctgggagaaa	300
atgacggaat ctgtgcagag tcacatcgcc tcgctgaact ggggctaccg cgtagctctc	360
cgggagaaaa aggtcgtcta tgagaatgct tacgggaggt tcattgggtc tcacaggatt	420
gtggcgacaa ataacaaagg taaagaaaa atctattcag cagagcggtt cctcatcgcc	480
acaggtgaga ggccccgcta cctgggcatc cctggagaca aagagtactg catcagcagt	540
gatgatcttt tctccttgcc ttactgcccg gggagagccc tagtagttgg tgcattcctat	600
gtgccttgg aatgtgcagg atttctggct ggtatcggt tagacgtcac tgtaattgtg	660
cggctccattc tccttagagg atttgaccaa gacatggcca acaaatcgg tgaacacatg	720
gaagaacatg gtatcaagtt tataaggcag ttctgtccaa cgaaaattga acagatcgaa	780
gcaggaaacac caggccgact cagggtgact gctcaatcca caaacagcga ggagaccata	840
gagggcgaat ttaacacagt gttgctggcg gtaggaagag attctgttac gagaactatt	900
ggcttagaga ccgtgggctg gaagataaac gaaaaaccg gaaagatacc cgtcacggat	960
gaagagcaga ccaatgtgcc ttacatctac gccatcggtg acatcctgga ggggaagcta	1020
gagctgactc ccgtagccat ccaggcgggg agattgctgg ctacagaggct gtatggaggc	1080
tccaatgtca aatgtgacta tgacaatgtc ccaacgactg tatttactcc tttggaatat	1140
ggctgttgtg gcctctctga agaaaaagcc gtagagaaat ttggggaaga aaatattgaa	1200
gtttaccata gtttcttttg gccattgaa tggacagtcc catcccggga taacaacaaa	1260
tgttatgcaa aaataatctg caaccttaaa gacgatgaac gtgtcgtggg cttccacgtg	1320
ctgggtccaa acgctggaga ggtgaacgag ggctttgcgg ctgcgctcaa gtgtgggctg	1380
actaagcagc agctggacag caccatcgcc atccaccgg tctgtgcaga gatattcaca	1440
acgttgtcag tgacgaagcg ctctggggga gacatcctcc agtctggctg ctga	1494

<210> SEQ ID NO 35
 <211> LENGTH: 1578
 <212> TYPE: DNA
 <213> ORGANISM: mus musculus

<400> SEQUENCE: 35

atggcgccga tggtagggcg gatggtggcg gcgctgcgtg gaccacgacg gcgcttccgg	60
ccgcggacac gggctctgac acgcgggaca aggggcgcgg cgagtgcagc gggagggcag	120
cagagctttg atctcttggt gatcgggtgg ggatccggtg gcctagcttg tgccaaggaa	180

US 8,574,869 B2

107

108

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gctgctcagc tgggaaagaa ggtggtgtg gctgactatg tggaaccttc tccccgaggc	240
accaagtggg gccttggtgg cacctgtgtc aacgtgggtt gcatacccaa gaagctgatg	300
catcaggctg cactgctggg gggcatgac agagatgtc accactatgg ctgggaggtg	360
gcccagcctg tccaacacaa ctggaagaca atggcagaag ccgtgcaaaa ccatgtgaaa	420
tccttgaaact ggggtcatcg cgtccaactg caggacagga aagtcaagta ctttaacatc	480
aaagccagct ttgtggatga gcacacagtt cgcggtgtgg acaagggcgg gaaggcgact	540
ctgctttcag ctgagcacat tgtcattgct acaggaggac ggccaaggta cccacacaaa	600
gtcaaaggag ccctggaata tggaatcaca agtgacgaca tcttctggct gaaggagtcc	660
cctgggaaaa cgttggtggt tggagccagc tatgtggccc tagagtgtgc tggcttcttc	720
actggaattg gactggatac cactgtcatg atgacgagca tccctctccg aggctttgac	780
cagcaaatgt catctttggt cacagagcac atggagtctc atggcaccca gttcctgaaa	840
ggctgtgtcc cctcccatc caaaaaactc ccaactaacc agctgcaggt cacttgggag	900
gatcatgctt ctggcaagga agacacaggc acctttgaca ctgtcctgtg ggccataggg	960
cgagttccag aaaccaggac tttgaatctg gagaaggctg gcatcagtag caaccctaag	1020
aatcagaaga ttattgtgga tgcccaggag gctacctctg tccccacat ctatgccatt	1080
ggagatgttg ctgaggggag gcctgagctg acgcccacag ctatcaaggc aggaaagctt	1140
ctggctcagc ggctctttgg gaaatcctca accttaatgg attacagcaa tgttccaca	1200
actgtcttta caccactgga gtatggctgt gtggggctgt ctgaggagga ggctgtggct	1260
ctccatggcc aggagcatgt agaggtttac catgcatatt ataagccct agagttcacg	1320
gtggcggata ggatgcac acagtgtac ataaagatgg tatgcatgag ggagcccca	1380
caactggtgc tgggcctgca cttccttggc cccaacgtg gagaagtcac ccaaggattt	1440
gctcttggga tcaagtgtgg ggcttcatat gcacaggtga tgcagacagt agggatccat	1500
cccacctgct ctgaggaggt ggtcaagctg cacatctcca agcgtccgg cctggagcct	1560
actgtgactg gttgctga	1578

What is claimed is:

1. A method for the prevention of the reduction of a disulfide bond in an antibody expressed in a recombinant host cell, comprising, following fermentation, sparging the pre-harvest or harvested culture fluid of said recombinant host cell with air, wherein the amount of dissolved oxygen (dO₂) in the pre-harvest or harvested culture fluid is at least 10%.

2. The method of claim 1 wherein said air sparging is continued until the pre-harvest or harvested culture fluid is at least 30% saturated with air.

3. The method of claim 1 wherein said air sparging is continued until the pre-harvest or harvested culture fluid is between about 100% saturated to about 30% saturated with air.

4. The method of claim 1 wherein the amount of dissolved oxygen (dO₂) in the pre-harvest or harvested culture fluid is at least 30%.

5. The method of claim 1 wherein the antibody is a therapeutic antibody.

6. The method of claim 1 wherein the antibody is a biologically functional fragment of an antibody.

7. The method of claim 1 wherein the host cell is eukaryotic host cell.

8. The method of claim 7 wherein the eukaryotic host cell is a mammalian host cell.

9. The method of claim 1 wherein the host cell is prokaryotic host cell.

10. The method of claim 9 wherein the prokaryotic host cell is a bacterial cell.

* * * * *